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Morphology and Physiology of Morphologically
Unusual Bacteria.

by

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A thesis submitted in fulfilment of the
requirements for the degree of
Doctor of Philosophy.

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Abstract

The morphological and associated physiological aspects of the chemoorganotrophic and photoorganotrophic appendaged (prosthecate) bacteria were considered. Initially work centred on the enrichment, isolation and enumeration of the chemoorganotrophic species Ancalomicrobium, Caulobacter and Hyphomicrobium from freshwater sources which varied in eutrophication levels. Induction and repression of the multiappendaged form of an Ancalomicrobium isolate, induced by environmental stimuli, was indicated. Additionally the morphological diversity of Hyphomicrobium found to be expressed in response to carbon source variation (methanol to methylamine) brings into doubt the validity of generic classification on the grounds of morphology.

Of the appendaged photoorganotrophic (Athiorhodaceae) species isolated, the growth and reproduction of a Rhodomicrobium species was studied in detail. This microorganism was shown to be similar physiologically to Rh.vanniellii (Duchow and Douglas, 1949) but differed in that it produced exospores in profusion during the stationary phase. The formation, germination and physiological characteristics of these resting cells were examined. Information from the growth and reproduction studies, correlated with ultrastructural work, was used to formulate a model for growth and replication applicable to the vegetative cell and exospore.

The obligatory, sequential, differential events required for growth and replication of the Rhodomicrobium

swarm cell lead to exploitation of this system as a model for the study of differentiation. A selective synchronisation procedure was formulated and the resulting synchronised swarm cell population characterised morphologically and physiologically with respect to the differential cycle.

Contrary to reports in the literature no extrachromosomal (plasmid) DNA could be detected in any of the appendaged, obligately life cycled genera. In addition, subdivision of these genera by reference to their mole percent G + C content was found to be of little value.

ACKNOWLEDGEMENTS

I should like to extend my gratitude and thanks to my supervisor, Prof. R. Whittenbury, not only for his expert guidance and advice, but for his continual encouragement, enthusiasm and friendship. In addition I am grateful to Dr. S.B. Primrose for most helpful discussion and technical direction in latter parts of my study. This work was carried out during the tenure of a Natural Environmental Research Council Studentship.

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DECLARATION

This thesis has been composed by myself and has not been accepted in any previous application for a degree. The results presented were obtained by myself under the supervision of Prof. R. Whittenbury.

Crawford S. Dow.

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MORPHOLOGY and PHYSIOLOGY of MORPHOLOGICALLY
UNUSUAL BACTERIA.

Section 1.

Introduction.

Morphology and Physiology of Morphologically Unusual

Bacteria.

Section 1. Introduction.

When Stanier and van Niel (1962) published their concept of a bacterium they described several major groups of bacteria, most of which fell into the precise morphological categories of spheres, rods, filaments and spirals. There are, however, notable exceptions to and complications of these morphologies, the most interesting and bizarre of which are the shapes of prosthecate bacteria.

Prosthecae have been defined by Staley (1968) as: "semi-rigid appendages extending from a procaryotic cell with a diameter which is always smaller than that of the mature cell and which are bounded by the cell wall".

Prior to this definition cellular extensions had been referred to as stalks, filaments or hyphae depending on the bacterial genera under study. They bear the most important feature in that the prosthecae are integral parts of the cell, not extracellular but part of the functional unit, i.e. they are extensions of the cell delineated by the cell membrane and wall. Caulobacter, Hypomicrobium and Rhodomicrobium are well known genera which meet the prosthecate criteria. Others such as Prosthecomicrobium, Ancalomicrobium and Prosthecochloris have only recently been described and characterized (Staley, 1968; Gorlenko, 1970).

Microbiologists in the past decade have devoted a great deal of their intellect and enthusiasm to the study of one organism, namely Escherichia coli. Although it is clearly advantageous to study a single microbial type the preoccupation with the essential details of this model has unfortunately displaced attention from bacteria which are more diverse (Starr and Skerman, 1965).

This thesis is not presented as a study of bacterial diversity per se. Consideration will be given to the physiology and morphology of Prosthecae bacteria but attention will be primarily focussed on model, synchronised cell systems with the potential to answer questions at both the micro and macromolecular level in the spheres of differentiation, de novo cell growth and cell mortality for which non-prosthecae bacteria are wholly unsuited as experimental material.

They bear no relationship, however, structurally or physiologically to the recently described spore appendages (Hodgkiss et al, 1967; Samsonoff et al, 1970) or the bacterial spines characterised by Easterbrook et al (1973).

Literature

Easterbrook, K.B., McGregor-Shaw, J.B., McBride, R.P. (1973) Ultrastructure of bacterial spines. Can. J. Microbiol., 19, 995-997.

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Samsonoff, W.A., Hashimoto, T., and Conti, S.F. (1970) Ultrastructural changes associated with germination and outgrowth of an appendage-bearing clostridial spore. J. Bact., 101, 1038-1045.

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Section 2.

Obligate Chemoorganotrophic Appendaged Budding Bacteria,
Ancalomicrobium, Prosthecomicrobium, Caulobacter and
Hyphomicrobium.

Section 2.

Obligate chemoorganotrophic appendaged budding bacteria, Ancalomicrobium, Prosthecomicrobium, Caulobacter and Hyphomicrobium.

Section 2.1 Introduction.

Attempts to classify the appendaged (stalked or prosthecate) bacteria have encountered considerable problems, primarily because of their diverse morphological and physiological characteristics. Jones (1905) was the first to observe Caulobacter and described the stalk as "a long single polar flagellum". Later Henrici and Johnson (1935) defined the stalk as a material "secreted from one side or one end" of the cell comparable with the ferric hydroxide secretion of Gallionella (Van Iterson, 1958; Poindexter and Lewis, 1966) or gum of Nevskia and Siderorhacus. Not until the detailed ultrastructural studies of Houwink (1952) and Poindexter (1964) was it confirmed that the stalk of Caulobacter is an integral part of the cell, the cell wall and cytoplasmic membrane being continuous with those of the cell proper.

Asticcacaulis, a genus described by Poindexter (1964) is identical to Caulobacter except that the stalk is excentral and lacks adhesive material at its tip. This is positioned at the base of the cell as in Caulobacter (figure 2.1). Pate and Ordal (1965) proposed that the Asticcacaulis stalk be termed a 'pseudo-stalk' on the basis that it is not involved in attachment. There seems no justification for adopting a different terminology for two identical structures. The holdfast material is probably extruded centrally therefore a mere shift of the wall site of stalk production could account

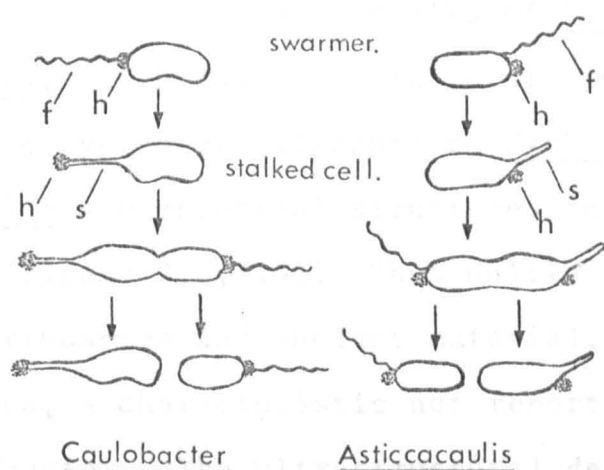


Figure 2.1

A semidiagrammatic representation of cellular differentiation and division in bacteria of the family Caulobacteraceae.

h Holdfast
f Flagellum
s Stalk

bands are found irregularly along the stalk (Schmidt and Stanier, 1966; Jones and Schmidt, 1973; Staley and Jordan, 1973). No analogous structures are found in the hyphae of Rhodomicrobium or Hyphomicrobium. The only common characteristics, apart from as yet undetermined physiological functions, is the integral cellular nature of both stalks and hyphae i.e. they are extensions of the cell wall and contain membrane and cytoplasm.

When originally isolated Rhodomicrobium was considered to be the photosynthetic counterpart of Hyphomicrobium because of their apparent morphological similarities (van Niel, 1954; Duchow and Douglas, 1949). Physiologically, however, they are quite distinct, Hyphomicrobium is an aerobic chemoorganotroph and Rhodomicrobium is an anaerobic photoorganotroph. Gross morphology and ultrastructure also differs. These aspects will be dealt with when the morphology and life cycle of Rhodomicrobium is considered (section 3.3).

for the excentral stalk not possessing a holdfast.

In contrast to the cellular stalks of Caulobacter and Asticcacaulis, which have no structural function in reproduction, the hyphae or filaments of Hyphomicrobium and Rhodomicrobium are essential structures in daughter cell formation (figure 2.2,2.3). Thus unlike stalks, hyphae contain ribosomes and nuclear material. In addition hyphae can branch, a characteristic not reported for the stalks of Caulobacter. The ultrastructural details of hyphae and stalks show other differences. Caulobacter possesses a unique membranous organelle which is claimed to be the origin of a complex membrane system found within the stalk (Cohen-Bazire et al, 1966). Characteristic cross bands are found irregularly along the stalk (Schmidt and Stanier, 1966; Jones and Schmidt, 1973; Staley and Jordan, 1973). No analogous structures are found in the hyphae of Rhodomicrobium or Hyphomicrobium. The only common characteristics, apart from as yet undetermined physiological functions, is the integral cellular nature of both stalks and hyphae i.e. they are extensions of the cell wall and contain membrane and cytoplasm.

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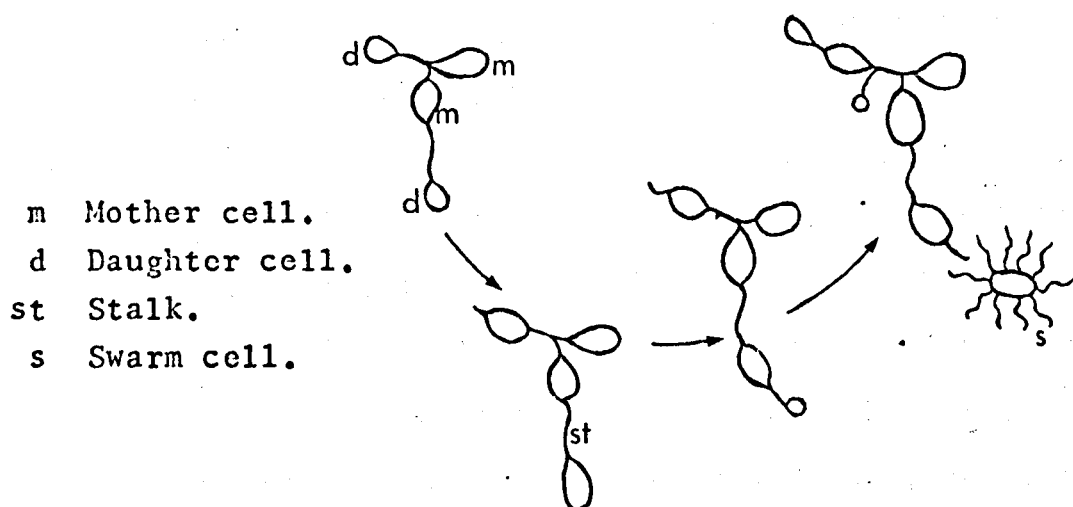


Figure 2.2 Rhodomicrobium

Vegetative growth of Rhodomicrobium.

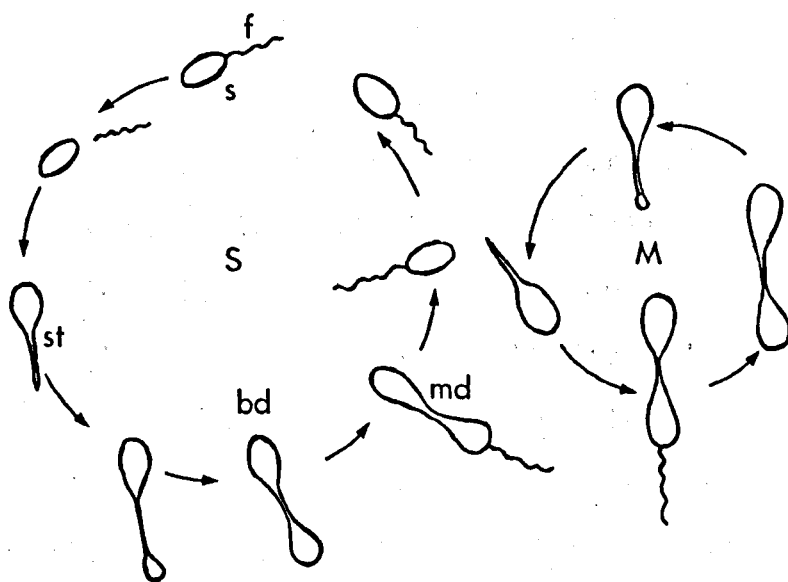


Figure 2.3 Hyphomicrobium

Vegetative growth of Hyphomicrobium.

- s Swarm cell.
st Stalked cell.
bd Bud formation.
md Mother-daughter cell.
f Flagellum.
S Swarm cell cycle.
M Mother cell cycle.

The type species of Hyphomicrobium is H. vulgar Stutzer and Hartleb (1899) which has been studied both morphologically and physiologically (Mevius, 1953; Zavarzin, 1961; Hirsch and Conti, 1964 a and b; Tyler and Marshall, 1967 a and b; Tyler, 1970). The isolation of other strains (Leifson, 1964; Hirsch, 1968; Tyler, 1970) has heightened controversy and doubt over the validity of other stalked or hyphaed genera which have been observed but incompletely characterised. In 1967 Tyler and Marshall demonstrated the involvement of Hyphomicrobia in the oxidation and deposition of manganese in freshwater pipelines. The bacterium responsible for the manganese deposition studied by Tyler and Marshall strongly resembles the manganese oxidizing soil organism Pedomicrobium. This genus was described by Aris-tovskaya (1961) as an ovoid cell which multiplies by budding from the tips of 3-4 filaments which grown out from one cell or occasionally from the mother cell directly. After studying the pleomorphy of Hyphomicrobium Tyler and Marshall, (1967b) concluded that Pedomicrobium was but one expression of the morphological diversity of Hyphomicrobium (figs.2.4 and 2.5). Further evidence of this contention has been presented (Bauld et al, 1971; Bauld and Tyler, 1971; and this thesis) and it now appears wholly justifiable to regard Pedomicrobium as synonymous with Hyphomicrobium.

Pograntz (1957) described a parasitic microorganism isolated from human nasal mucous as morphologically similar to Hyphomicrobium but differing sharply in physiology. On the strength of his findings he proposed the new genus Hyphomonas. Starr and Skerman (1965) assimilated and



Figure 2.4 Hyphomicrobium

Hyphomicrobium showing intercalary cells narrowed at both ends and multiple branching of filaments. (Tyler and Marshall, 1967)

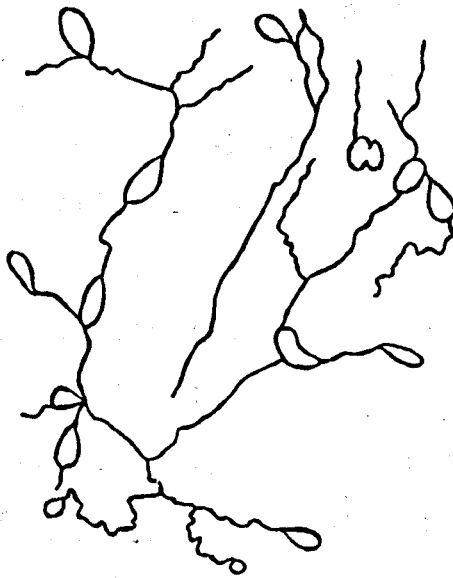


Figure 2.5 Pedomicrobium

A portion of a colony after the metallic oxides were dissolved. Schematic drawing made from a photograph of Aristovskaya (1961).

reviewed the data available on Hyphomonas polymorpha and Hyphomicrobium. They concluded that it would be nonsensical to separate two genera on the grounds of non-existent differences in nutrient status, (these arose because of misinterpretation of the literature) or unproven claims of parasitism.

Zavarzin (1961) recognized nine genera of budding stalked or hyphaed bacteria when he described a classification on the basis of cell division by other than binary fission (in itself a false basis). Subsequent work has shown some of these genera to be invalid, e.g. Pedomicrobium and Hyphomonas or artifacts, e.g. the genera Klassilnikoviae (Kriss and Mitzkerich, 1959) was, in fact the colloblasts of Ctenophore tentacles damaged by the sampling technique.

Zavarzin (1961, 1964, 1968) documented the genus Metallogenium. This microorganism forms radiating filaments coated with manganese oxide and reportedly has four stages to its life cycle: a unicellular motile stage, a multicellular stage with radial filaments and two zoogloeic stages which consist of tightly twisted intertwined filaments (figure 2.6). Although originally suggested to be related to Hyphomicrobium (Zavarzin, 1961) the apparent plasticity and size variability of Metallogenium makes this doubtful. There are more definite similarities in morphology and reproduction between Metallogenium and the myxoplasm (Skerman, 1967; Schmidt, 1971).

Henrici and Johnson (1935) when studying 'attaching' lake bacteria by their submerged slide technique described most of the genera named above. In addition they detailed

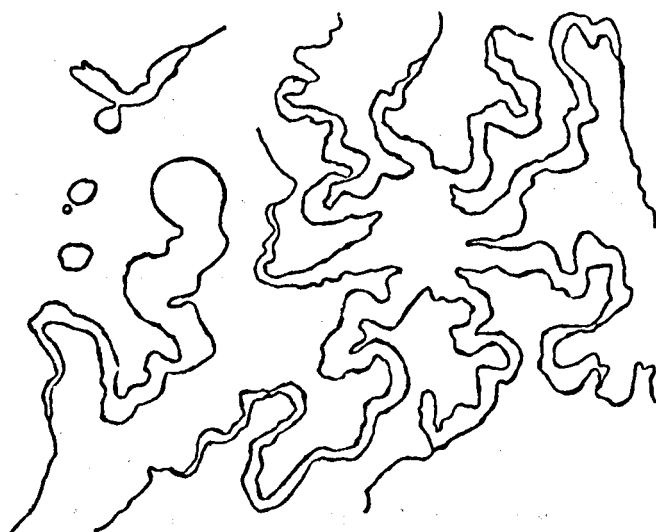


Figure 2.6 Metallogenium symbioticum

Schematic representation of several stages of the life cycle of Metallogenium symbioticum.

Budding, motile buds, a round body which sprouts twisted filaments and microcolonies are shown.

(Zavarzin, 1961)

the genus Blastocaulis. The organisms of this genera are planktonic freshwater bacteria, spherical, oblong or pear shaped cells with long slender stalks which in the mature state resemble Hyphomicrobium (Starr and Skerman, 1965). The stalks are often attached to a common holdfast and multiplication is characteristically by budding (figures 2.7, 2.8, 2.9). Only one species has been successfully cultured (Staley, 1973a) but several workers have confirmed these observations (Fott and Komarek, 1960; Haltobagyi, 1965; Hirsch, 1968-1970 unpublished). Pfennig (pers. comm.) has assimilated the published and unpublished data on the two genera Blastocaulis and Planctomyces and has shown one to be synonymous with the other arguing for retention of the more descriptive Blastocaulis as the genetic name.

Staley (1968) in a recent study of freshwater bacteria has focused attention on microorganisms found in open water (other than the "attaching bacteria") with the discovery of previously observed (Stefanov and Nikitin, 1965) but uncharacterized procaryotes with numerous cellular appendages. Direct electron microscopic preparations of concentrated water samples revealed nine morphologically distinct forms, most possessing multiple appendages. Two new genera, Prosthecomicrobium and Ancalomicrobium were proposed to accomodate the isolated strains. Prosthecomicrobium (figure 2.10) have appendages, (prosthecae as defined by Staley, 1968) tapering to a blunt tip and extending in all directions from the cell. The ratio of prosthecae length to cell diameter is normally less than one and reproduction is by binary fission. The phototrophic green sulphur bacterium Prosthecochloris isolated and characterised by Gorlenko (1970)

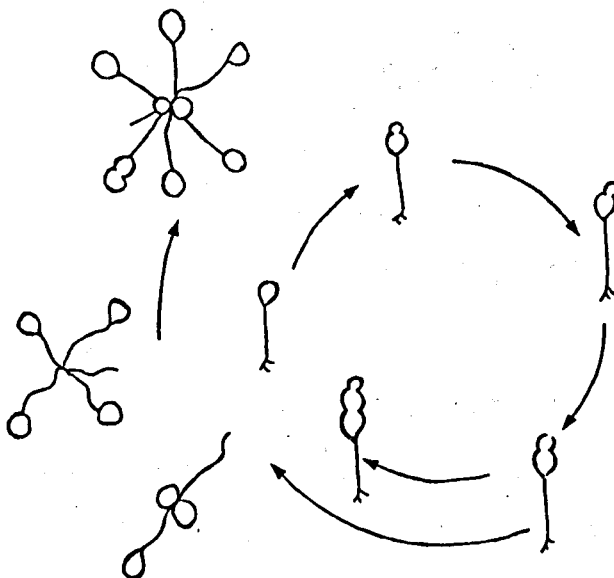


Figure 2.7 Blastocaulis

Life cycle of Blastocaulis. Schematic drawing made from the drawings and description of Fureva (1953) by Zavarzin (1961).

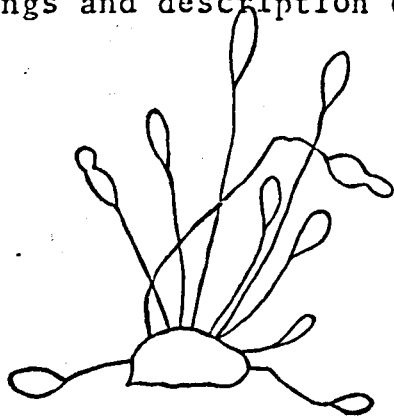


Figure 2.8 Blastocaulis

Blastocaulis species as drawn by Henrici and Johnson (1935).

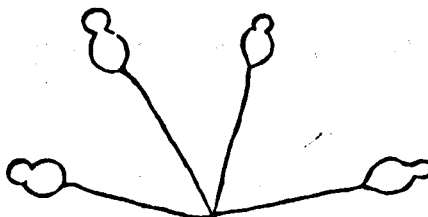


Figure 2.9 Bl. sphaerica

Blastocaulis sphaerica as drawn by Henrici and Johnson (1935).

is comparable morphologically with Prosthecomicrobium. In contrast Ancalomicrobium (figure 2.11) has from two to eight prosthecae which attain a length of approximately three times the diameter of the cell. These prosthecae may have bifurcations but do not bear buds. Cell reproduction is by budding, the buds forming at one site on the cell body. Both genera lack holdfast material and only Prosthecomicrobium has been found to be motile.

Where as Staley (1968) and Gorlenko (1970) have documented conclusive evidence of the physiology and morphology of their isolates, other workers (Stefanov and Nikitin, 1965; Volarovich and Terent'ev, 1968) have studied by direct electron microscopic techniques, soils, peats, salt and freshwater with concomitant publication of electron micrographs of morphologically diverse bodies, with the proviso, " we will consider only types of bodies which can be assumed to be biological in nature in view of the complexity and degree of differentiation of their fine structure" so hopefully averting the question animal, vegetable or mineral? Several of the organisms reported by Volarovich and Terent'ev (1968) and Stefanov and Nikitin (1965) do bear marked similarities to the defined isolates of Staley (1968) and can most probably be regarded as multiappendaged procaryotes.

Of the prosthecate genera few lack a polymorphic life cycle, Prosthecomicrobium and Ancalomicrobium reportedly possess relatively simple cell cycle characteristics but genera such as Hynhomicrobium, Rhodomicrobium and Caulobacter all shown inherent diversification. A new

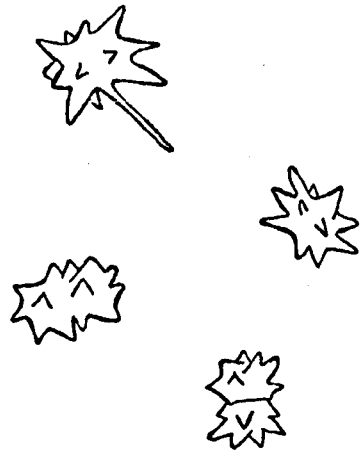


Figure 2.10 Prosthecomicrobium

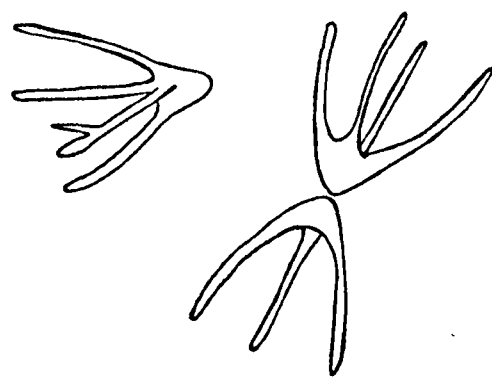


Figure 2.11 Ancalomicrobium

genus of the Actinomycetales, Geodermatophilus (Leudemann, 1968) has been studied by Ishiguro and Wolfe (1970) and shown to express to a considerable degree morphogenesis and differentiation during the life cycle (figure 2.12). The R-form of this microorganism multiplies exclusively by budding. The mother cell and daughter cell are joined by a tubular structure (filament or hyphae), daughter cells developing as polar or subpolar outgrowths comparable with other appendaged genera. The parent retains the tube after detachment of the consistently smaller daughter cell.

Other microorganisms which might possibly be prosthecate include Kuznetsovia polymorpha and Caulococcus manganifer (Perfil'ev and Gabe, 1965), Helicoidal polyspheroides (Orenski et al, 1966 a and b), Agrobacterium polyspheroidum and Tuberoidobacter (Nikitin, 1970). Although these organisms have been precisely named they have only been tentatively described and nothing conclusive is known about their growth and physiology.

The described genera, although diverse in morphology when considered as a group (which may be questionable) have several characteristics in common. The principle source of isolates has been freshwater. All possess cellular appendages. Reproduction is invariably by budding i.e. polar growth (Whittenbury and McLee, 1967). The vast majority have a polymorphic life cycle. Several genera possess holdfast material and most are capable of assimilating C_1 compounds (Whittenbury, pers. comm.). There are numerous pertinent questions arising from any one of these characteristics. However, the basic fundamental questions are whether these organisms evolved to occupy ecological niches where survival advantage arose from these types of

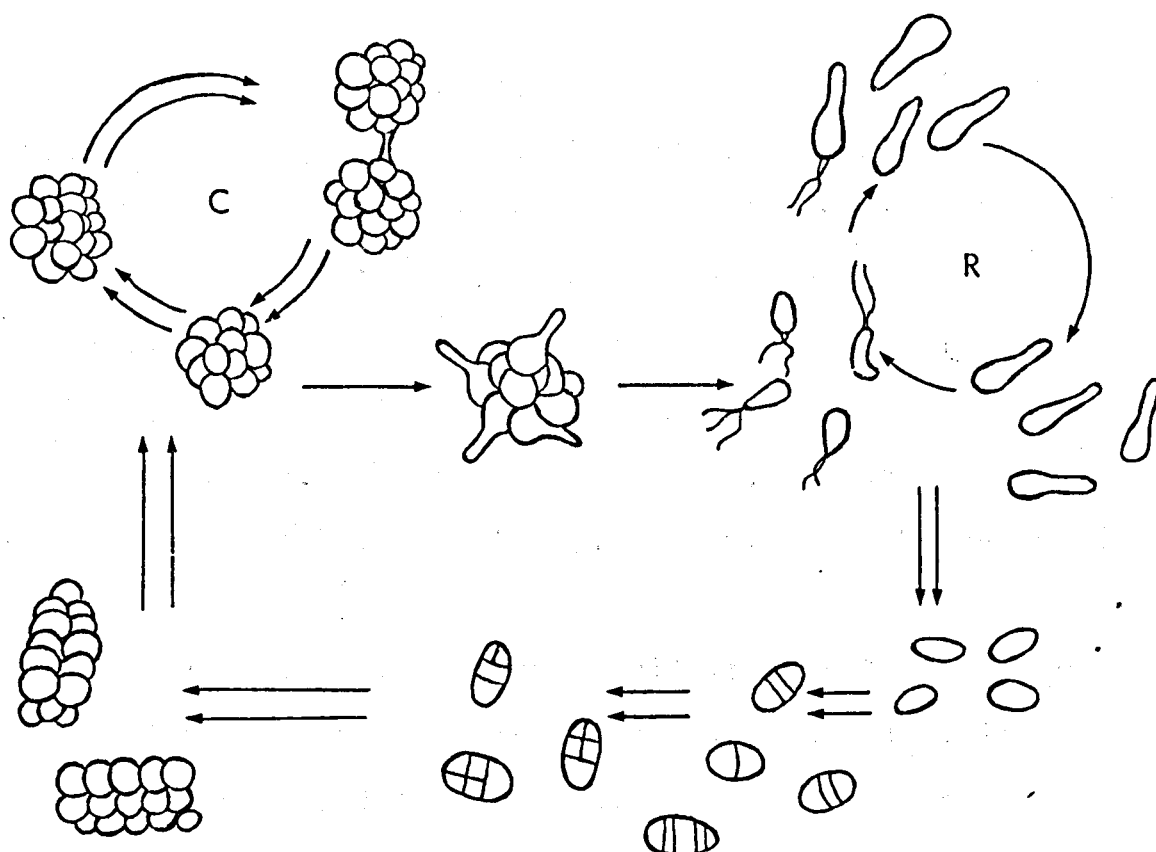


Figure 2.12 Geodermatophilus

Morphogenetic growth of Geodermatophilus.

C Multiplication cycle of form C.

R Multiplication cycle of form R.

→ Growth without M factor.

⇒ Growth with M factor.

(Ishiguro and Wolfe, 1970)

morphological and physiological developments and what specific advantages these developments confer.

This work was instigated with a view to studying the inherent morphological diversity of appendaged bacteria by the isolation of new and known species. Correlation of morphological diversity and viable numbers with the chemical environment, i.e. eutrophication levels, was attempted, the possibility of a biological indicator organism being considered.

Species similar in many details to Ancalomicrobium were isolated and the inherent morphological diversity studies and extrapolated to genera which have been described in the literature. The complex pleomorphy and growth cycle of Hyphomicrobium was also investigated.

Assimilation of the information obtained shows that the credibility of several named genera is in doubt. In context the validity of morphology as a specific generic marker is severely questioned.

Section 2.2 Materials and Methods.

a) Freshwater bodies sampled.

i) Loch Leven, Kinross, Scotland.

Loch Leven, the principle sample site, was chosen because it is a shallow, nutrient rich freshwater body (area 13.3 km², mean depth 3.9m). Chemical data dates back to 1963 but an intensive study instigated in 1966 as part of the International Biological Programme has enhanced details available on chemical and related biochemical changes.

ii) Harperigg Reservoir, Midlothian, Scotland.

Situated in the Pentland Hills, Harperigg Reservoir is a relatively shallow, oligotrophic freshwater body isolated from industrial and excessive agricultural pollution. It comes under the auspices of the Midlothian Water Board.

iii) Lake Windermere, Westmorland.

Conveniently divided into two well separated basins, Windermere North and Windermere South, Windermere presents an industrially and agriculturally polluted eutrophic sample site (North) and an oligotrophic sample site (South). Additionally the Natural Environmental Research Station at Sawrey has compiled a data bank on both basins. Consequently sampling and analysis were simplified.

b) Sampling techniques

Water samples were collected aseptically and maintained at a constant temperature (4°C) prior to analysis which was normally undertaken within one hour.

i) Surface samples.

Sterile 500 ml glass stoppered bottles were immersed to a depth of between three and four inches and 200 ml samples collected.

ii) Shallow mud samples.

Sterile wide mouthed chemical bottles were used to scoop surface mud samples from sites at a depth of one to two feet.

iii) Deep water samples.

For the purpose of obtaining water samples from a profile depth series a 1 litre Friedinger water bottle was used (Hans Buchi, Berne, Switzerland).

iv) Deep water mud samples.

Mud samples were obtained by means of a Jenkin surface mud sampler which takes a mud core approximately 30 cm. in depth, with the overlying water in situ. There is some slight disturbance of the surface mud during its enclosure in the sample tube but not enough to make any significant difference to the sample contents. (This apparatus can be obtained from The Lakes Instrument Co.Ltd., Oakland, Windermere, Westmorland)

c) Enrichment and Isolation

Media

Mineral base

NH_4Cl 0.5 g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g

NaCl 0.1 g

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05g

Trace elements solution 10 mls.

Distilled water to 1 litre.

After autoclaving, KH_2PO_4 was added aseptically to the desired concentration.

Pfennig and Lippert's trace element solution (Pfennig and Lippert, 1966).

Vitamin solution (Staley, 1968)

biotin	2	mg
folic acid	2	mg
pyridoxine HCL	10	mg
riboflavine	5	mg
thiamine HCL	5	mg
nicotinamide	5	mg
calcium pantothenate	5	mg
B ₁₂	0.1	mg
p-amino benzoic acid	5	mg
Distilled water to 1 litre.		

This solution was sterilized by membrane filtration and added aseptically after autoclaving.

Medium A contained per litre 20 mls mineral base, 100 mg peptone and 100 mg yeast extract.

Medium G+Y contained per litre, 20 mls mineral base, 100 mg glucose and 100 mg yeast extract.

Medium G + CA contained per litre, 20 mls mineral base, 100 mg glucose, 100 mg vitamin free casamino acids and 10 mls vitamin solution.

Medium P contained per litre, 100 mg peptone plus 10 mls vitamin solution.

Medium II contained per litre, 20 mls mineral base supplemented with 0.01m methanol, formate, formamide or methylamine HCL which were added aseptically after autoclaving.

Medium IIV contained per litre, 20 mls mineral base plus 5 mls methanol which was added aseptically after autoclaving.

Medium AII (Attwood and Harder, 1972)

KH ₂ PO ₄	1.74	g
NaH ₂ PO ₄ 2H ₂ O	1.38	g
(NH ₄) ₂ SO ₄	0.5	g
MgSO ₄ 7H ₂ O	0.2	g

pH adjusted to 7.0 with N NaOH.

After autoclaving 0.025 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.5 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2g KNO_3 and 5 ml methanol were added aseptically.

All solid medium contained 1.5% (w/v) Bacto Difco agar. Plates of agar medium were dried in a low heat oven and used immediately.

i) Prosthecomicrobium, Ancalomicrobium and Caulobacter.
Enrichment.

As quickly as possible after collection 1 ml and 100 ml freshwater samples were used to inoculate 100 ml medium.

Media P, A, G+Y and G+CA were used routinely. All flasks were incubated in the dark at room temperature and at 30°C either statically to allow pellicle formation or on a rotary shaker (approximately 150 rev/min.) to increase aeration.

Isolation.

Samples from all flasks were examined regularly by phase contrast microscopy. When sufficient growth had occurred, generally after 2 - 5 weeks, or the desired bacteria were present in sufficient numbers (relative proportion of about 1 in 10 or 20 cells) serial dilutions were made and inoculated onto the surface of solid medium.

Incubation was at room temperature and at 30°C for 2 - 4 weeks.

Individual colonies were picked using sterile tooth-picks and inoculated onto the surface of fresh agar medium, i.e. a patch plate technique. After a further period of incubation the individual patches were examined microscopically and the organisms of interest isolated and purified by streaking.

An alternative, direct method of isolation involved centrifuging 25 ml samples at 3,000g for 15-25 minutes. The bacterial pellet was resuspended in 2 ml of sample water, serially diluted and the spread plate, patch plate technique employed.

Culture and maintenance of isolates.

Liquid cultures were grown aerobically in 250 ml conical flasks containing 75 mls medium. Incubation was on a rotary shaker at 30°C in the dark.

Agar plates were incubated aerobically at 30°C. Slopes were prepared in ordinary 5/8" test tubes and dried at 37°C overnight prior to inoculation.

All Caulobacter cultures were grown on slopes of G + Y medium. The Ancalomicrobium isolates were maintained on slopes of G + Y and A medium. Cultures were transferred every six to seven weeks, incubated at 30°C until reasonable growth occurred and then stored at 4°C.

ii) Hyphomicrobium

Enrichment.

5.0ml water or 0.5g mud was inoculated into 100ml HY medium contained in a 250 ml Quickfit flask which was then sealed with a rubber Suba-seal to ensure an enclosed atmosphere.

The cultures were incubated statically at 30°C in the dark for 2 - 5 weeks.

A secondary enrichment method involved preparation of the sample by centrifugation as described above for Ancalomicrobium, Prosthecomicrobium and Caulobacter. 0.1ml samples were inoculated into agar medium containing only

mineral salts, i.e. HY medium lacking methanol. A methanol saturated filter paper was then placed in the lid of each plate. Incubation at 30°C was carried out in sealed Tupperware containers to minimise the loss of methanol vapour.

Isolation.

Liquid cultures were examined microscopically, plated onto solid agar medium and the spread plate, patch plate method of isolation used. Patch plates were also prepared directly from the solid medium enrichments.

Culture and Maintenance of isolates.

Liquid cultures were grown in Quickfit flasks sealed with Suba-seals to prevent the loss of methanol. Incubation was at 30°C on a rotary shaker. A high aeration rate was found essential for rapid growth of Hyphomicrobium on HY medium, consequently small volumes (25-50mls) of medium were used.

Rapid growth on AH medium was ensured by flushing the system with oxygen free nitrogen. Aggitation was maintained to ensure adequate suspension and dispersion of the culture. When using AH medium in a sealed system it was essential to release the pressure generated by the reduction of KNO_3 to N_2 .

Aerobic plate cultures were incubated at 30°C in sealed Tupperware containers. Plates for anaerobic culture were incubated in nitrogen filled anaerobic jars at 30°C.

Hyphomicrobium isolates were maintained on slopes of HY and AH medium in sealed 25 ml screw cap bottles. Cultures remained viable under such conditions for three to four months.

d) Microscopy

i) Light Microscopy

Since fixing and staining resulted in cell distortion, all cultures were examined by phase contrast microscopy using a Leitz Orthoplan microscope fitted with an Orthomat camera unit.

Interference photomicrographs were obtained using a Leitz interference condenser and lense system.

Photomicrographs

Initially photomicrographs were taken on Ilford FP4 (ASA 125) film developed as per the manufacturers specifications. Experimentation showed, however, that Kodak Panatomic X (ASA 32) gave a finer grain, greater contrast, and therefore better definition. Films were developed in Kodak D 19 developer for 2 minutes, or alternatively for 8 minutes in Paterson's Acutol, and fixed in Ilford Hypan rapid fixative.

Slide culture.

The methods of culturing microorganisms under the light microscope have been reviewed by Quesnel (1969). However, the majority of these are complex and require intricate handling of apparatus and materials. The following method was devised to allow rapid assessment of microbial growth and physiological requirements and was found to be adequate under both aerobic and anaerobic conditions.

Slides and coverslips were cleaned by immersion in chromic acid for 24 hrs., washed with distilled water and sterilised in absolute ethanol.

A small drop of molten agar medium was put onto a slide and a coverslip applied immediately. When the system was to be anaerobic care was taken to ensure the exclusion of air bubbles. After a few minutes the coverslip was gently removed using a fine pair of forceps, leaving a thin unbroken film of agar. In the case of aerobic cultures a trough was cut from the centre of the agar to ensure a sufficient reservoir of oxygen. A small drop of liquid culture, sufficient to give surface cover only, was then inoculated onto the agar and a sterile cover slip applied. Any protruding agar was removed and the system sealed with a Paraffin Wax: Vaseline mixture (1:1 w/w).

ii) Electron microscopy.

All preparations were observed in an AEI Corinth 275 electron microscope with a 50 μ aperture and an accelerating voltage of 60 kV.

Electron micrographs were taken on 70mm Ilford Line film, N4E50, which was developed in Ilford Phenisol and fixed in Kodafix.

Negative staining.

Droplets of a sample concentrate were placed on Parlodion-coated, 300 mesh, copper grids, fixed in OsO_4 vapour for 5 minutes and the excess moisture absorbed by filter paper. A drop of 1% (w/v) phosphotungstic acid (pH 7.0) or 0.5% (w/v) uranyl acetate (pH 4.5) was added and immediately absorbed with filter paper.

Shadowing.

Samples were prepared as for negative staining and shadowed in an AEI (EC9) coating unit with gold palladium at an angle of 15° .

Sections

The fixation procedure of Ryter and Kellenberger (1958) or the glutaraldehyde, acrolein, potassium permanganate method proposed by Hayat (1968) were followed for thin sections. Agar blocks were prepared, dehydrated with ethanol, transferred to propylene oxide and embedded in Araldite. (Araldite resin CY212, 4.9 gm; araldite hardener DDSA, 4.9gm; di-butyl phthalate DBT plasticiser, 0.075 gm; benzyl dimethylamine BDMA, 0.175 gm) Blocks were polymerized at 60°C for a minimum of two days.

Sections were cut with a glass knife on a Reichert "Om 12" ultramicrotome.

Sections were post-stained for 20 mins. in 0.5% (w/v) uranyl acetate, followed by 5 min. in 0.1% (w/v) lead citrate in 0.1N sodium hydroxide when required.

Section 2.3 Results and Discussion.

Although detailed ecological studies on this group of microorganisms are urgently required, the appendaged heterotrophic bacteria isolated and studied remained rather narrow, species of Caulobacter, Hyphomicrobium and Ancalomicrobium being considered primarily.

Enrichment.

The basis of the technique which has been found generally suitable for the enrichment of appendaged, budding, heterotrophic bacteria was devised by Houwink (1952) and only slightly modified by subsequent workers (Zavarzin, 1961; Poindexter, 1964; Hirsch and Rheinheimer, 1968; Staley, 1968). The published data on the enrichment and isolation of the required genera was reviewed and assimilated. Studies subsequently involved investigation and appraisal of these techniques with and without modification. The experience gained in the present work in conjunction with the information available in the literature suggests that consideration of the detail inherent in the following factors is of prime importance in enriching and isolating appendage heterotrophic bacteria from the environment.

i) Source

The majority of known appendaged bacteria have been observed in, and isolated from, freshwater environments low in organic nutrient content (Henrici and Johnson, 1935; Bowers et al, 1954; Zavarzin, 1961; Hirsch and Conti, 1964, a, b, 1965; Poindexter, 1964; Tyler and Marshall, 1967; Staley, 1968). Russian researchers have, however, reported the presence of appendaged bacteria in direct electron microscopic preparations of soils (Stefanov and Nikitin, 1965; Nikitin, 1973).

and peat of "natural humidity" from a variety of geographical locations (Volarovich and Terent'ev, 1968). Few isolations were attempted. Consequently extrapolation from and interpretation of these electron micrographs remains uncertain. In this study the majority of isolates were derived from water samples low in nutrient levels. Only Hyphomicrobium species were found in soil or mud enrichments.

ii) Nutrients

To prevent domination by other, more rapidly growing bacteria, carbon compounds, although used routinely for growth of pure cultures, were omitted from the primary liquid enrichments. The addition of low levels of peptone (1mg/litre) or simple carbon compounds e.g. methanol, formate, formamide or methylamine HCl at a concentration of 0.01 molar, in certain sample enrichments, however hastened growth of the required organisms (Table 2.1). The concentration of inorganic salts, within the range tested, other than phosphate, which can cause variation of stalk or filament length (Schmidt and Stanier, 1966; this thesis), has no obvious effect on enrichment populations occurring.

iii) Conditions of incubation

These were as described in the materials and methods section.

iv) Detection and recognition

Preparation and observation of samples by phase contrast microscopy allowed characterisation by recognition of the distinct morphological features of these genera.

Table 2.1 Medium and enriched genera.

Medium	ANCALO- MICROBIUM	T ^a	CAULO- BACTER	T ^a	HYPHO- MICROBIUM	T ^a
No added nutrients	+	6	++	3	+	6-7
P	++	4	+++	2	+	4
A	+	4-5	+++	2	+	4
G+Y	-		+	4	-	
G+CA	-		+	4	-	
H+formate	-		-		+	3
+formamide	-		-		+	3
+methylamine HCL	-		-		++	3
+ methanol	-		-		++	3
+ CH ₄	+	6	-		-	

T^a, incubation time in weeks.

- not present, + → +++ few to abundant

v) Isolation

Although tedious, the isolation procedures detailed in the materials and methods section were found to be the only way of achieving consistent success.

The isolates successfully obtained in pure culture are detailed in Table 2.2.

Occurrence and distribution.

Unlike 'typical' unicellular bacteria, each genus of heterotrophic appendaged bacteria contains individuals that are morphologically distinct and therefore recognisable. Based on number, size, shape and location of their appendages and the morphology of their cells certain individuals can be identified to the genus level simply by phase contrast microscopy. This allows the distribution of these genera in natural samples and their presence in enrichment cultures to be determined not only as to type but as to approximate percentages of the total microbial population, provided the following limitations are realised. Swarm cells, i.e. motile cells, do not possess stalks therefore not all individuals of a population are identifiable. Similarly, stalked Caulobacter cells are morphologically inseparable from non-budding Hyphomicrobium cells. Mistaken identity and underestimation of numbers are therefore the principle errors.

A microscopic study of the distribution of these genera demonstrated their ubiquitous presence in freshwater environments. No geographical or significant seasonal fluctuations of numbers were observed. However, the genera present varied with the depth from which the samples were drawn (Table 2.3). These findings for Hyphomicrobium and

Table 2.2

Number of pure cultures of appendaged
heterotrophic bacteria isolated from
various sample sites.

SAMPLE SITE	ANALO- MICROBIUM	CAULO- BACTER	HYPHO- MICROBIUM
Loch Leven	2	6	3
Harperigg		3	2
Windermere North		7	6
Windermere South		4	5
Various mud samples			2
Soil samples			1

Table 2.3

Appendaged heterotrophic bacteria detected
at various depths in Lake Windermere, North Basin
(September, 1971)

Depth (m)	<u>Hypnomicrobium</u>	<u>Caulobacter</u>	<u>Ancalomicrobium</u>
Surface	++	+++	+
0.2	+	+++	(+)
0.4	+	++	+
0.6	(+)	+	-
1	(+)	(+)	-
5	(+)	-	-
10	-	-	-
15	-	-	-
20	(+)	-	-
25	-	-	-
30	(+)	-	-
40	(+)	-	-
50	(+)	-	-
60	(+)	-	-

- not present

(+) rare

+ → +++ abundant

Ancaolomicrobium are comparable with those reported by Hirsch and Rheinheimer (1968).

The obligate aerobic heterotrophy of Caulobacter and Ancaolomicrobium the type strain, Ancaolomicrobium adetum, is a facultative anaerobe, (the isolates from this study were unable to grow anaerobically) explains their presence only in surface samples. The presence of Hypomicrobium in the oxygen depleted zones of Windermere can be explained by their ability to utilise nitrate in place of oxygen as a terminal electron acceptor i.e. denitrification (Sperl and Hoare, 1971; Attwood and Harder, 1972). Not all of the Hypomicrobium isolates cultured however were denitrifiers.

Estimates of the bacterial population.

Many studies have been made of the most suitable methods and viable count media for quantification of aquatic bacteria in natural environments. Medium composition (Floodgate, 1964; Carlucci and Pramer, 1957; Strzelazyk et al., 1969) experimental conditions (Buck and Cleverdon, 1960; Gunkel et al., 1961) and methods of estimation (Jannasch and Jones, 1959; Melchiorri-Sautolini and Cafarelli, 1967; Harris and Sommers, 1968) have all been shown to exert an effect on the count estimate. To avoid entanglement in the formulation of a 'general' medium the numbers of aquatic appendaged, heterotrophic bacteria in surface samples were estimated by specific plate enrichments and by direct microscopic observation so giving approximate numbers of individuals of any one genera (Table 2.4).

Direct microscopic counting showed that appendaged bacteria accounted for 0.5 to 0.6% of the total microbial population, a figure somewhat lower than that found by

Staley (1971) for the incidence of such microorganisms in a polluted stream (no indication was given as to the degree of pollution). In both direct and viable estimates Caulobacter species were the predominant genera followed by Nyphomicrobium species. The morphologically exotic Ancalomicrobium was encountered only infrequently suggesting that either the enrichment and counting techniques were inadequate or that the figure is a true reflection of a low population density in the natural environment. Alternatively one may only be counting a recognisable percentage of the viable entities of this genus i.e. this organism may possess a polymorphic life cycle with the exception that it is not obligate, as with Caulobacter, but environmentally controlled e.g. similar to Geodermatophilus (Ishiguro and Wolfe, 1970). Evidence for this will be presented.

Quantitative and qualitative variation in response to the environment.

Hirsch and Rheinheimer (1968) have reported inhibition of the growth of Nyphomicrobium by very low levels of amino acids and carbohydrates. Similarly, Caulobacter growth and morphology is correlated closely with the concentration of inorganic phosphate (Schmidt and Stanier, 1966). In addition morphological variation in Ancalomicrobium is considerable when organic nutrients are high (in excess of 200 ug/ml.) and inorganic phosphate concentration is varied (this thesis, page 50). Although the sample sites chosen, Harperigg reservoir, Loch Leven and Lake Windermere, represent a gradation from oligotrophic to eutrophic environments, no relationship between the chemical environment, organic or inorganic, and total numbers or morphology of

appendaged bacteria could be demonstrated (Table 2.4). It may be argued, however, that to observe any variation in numbers or morphology it may be necessary to go to greater extremes of oligotrophic and eutrophic conditions.

Ecological niché: aquatic or terrestrial?

The question as to whether these and similar genera are indigenous and specific to aquatic habitats was raised by Hirsch and Rheinheimer (1968) and answered in the affirmative. The advantages occurring from the distinct morphological features of these bacteria in aquatic environments can be easily seen (an increase in surface area facilitating increased uptake of nutrients, buoyancy, colonialism, polymorphic life cycles enhancing dispersal) but there is no evidence to suggest that microenvironments do not exist in terrestrial habitats where these organisms may grow and reproduce. Caulobacter (Poindexter, 1964) and Hynhomicrobium (Atwood and Harder, 1972) have been isolated from mud and terrestrial soils and there is no indication that these differ physiologically from aquatic isolates. In conjunction with the observations of the Russian researchers (Stefanov and Nikitin, 1965; Volarovich and Terent'ev, 1968) an indigenous and specific location for such genera can only be indicative of ecological nichés where the available nutrient concentrations are low, be it aquatic or terrestrial.

Anacalomicrobium isolates

The first description and the generic classification of this group of microroganisms is attributed to Staley (1963) who published electron micrographs of several strains found in freshwater in addition to culturing and further

Table 2.4

Number of viable cells per ml. as determined by spread plates.

Organisms Ancalomicrobium			Caulobacter		Hyphomicrobium
Media	P.	A.	P.	A.	H+ methanol
Loch Leven.					
vc/ml	2.0	1.0	200	170	16
TVC/ml	3×10^3	3.1×10^3	2.9×10^3	2.5×10^3	0.5×10^2
%	0.06	0.03	6.8	6.8	32
Harperigg					
vc/ml	1.0	1.0	150	180	6
TVC/ml	2.6×10^3	3×10^3	2.9×10^3	2.7×10^3	0.4×10^2
%	-	-	5	6.5	15
North Windermere					
vc/ml	1.0	1.0	250	320	20
TVC/ml	3.4×10^3	3.4×10^3	3×10^3	3×10^3	1×10^2
%	-	-	8	10.5	20
South Windermere					
vc/ml	1.0	1.0	160	210	8
TVC/ml	2.5×10^3	2.5×10^3	2.7×10^3	2.6×10^3	0.7×10^2
%	-	-	6	7.5	11

vc., viable count; TVC, total viable count; %, $\frac{vc}{TVC} \times 100 / 1$

characterising one isolate, the type strain, Ancalomicrobium adetum. This is a unicellular gram negative bacterium having from two to eight appendages extending from the cell, attaining 3-4 μ in length at maturity. Reproduction is by budding (appendages do not bear buds) and division occurs transversely when the mother and daughter cells have attained approximately the same size.

During the course of isolation attempts three species of Ancalomicrobium were encountered. Two were successfully isolated from dilute peptone enrichments but the third was only observed in methane enrichments (composed of a mineral base plus 5%(v/v) gaseous methane in the atmosphere) and never attained numbers great enough for isolation attempts to be practicable.

Methane 'utiliser'

Observation of the 'methane' appendaged microorganism, all be it in enrichments, showed that reproduction was by budding from one of the four to six cellular appendages (Plates 2.1,2.2,2.3) bearing in this respect a resemblance to one of the electron micrographs (figure 7) published by Staley (1968). Although no further characterisation could be attempted, it is worthy of note that this organism presumably assimilates C_1 material (since it remains viable after several transfers in the CH_4 system) and may therefore correspond physiologically to the majority of appendaged bacteria so far studied (Whittenbury, pers.comm.).

Ancalomicrobium: morphology

The two isolates obtained in pure culture were morphologically similar when observed by phase contrast (Plate 2.4,2.5) but markedly different when studied by

Phase contrast
(X1120) of
Ancalomicrobium
species observed in
methane enrichments.

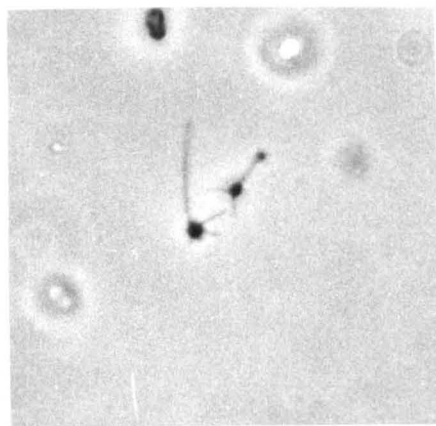


Plate 2.1

Primary methane enrichments
of Ancalomicrobium species

Phase contrast x 1120

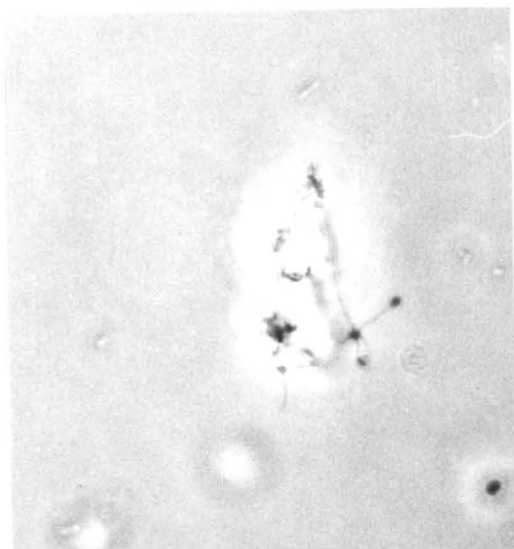


Plate 2.2

Primary methane
enrichments of
Ancalomicrobium
species.

Phase contrast x 1120

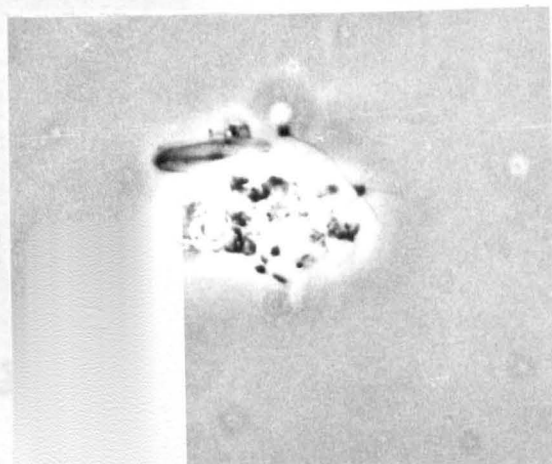


Plate 2.3

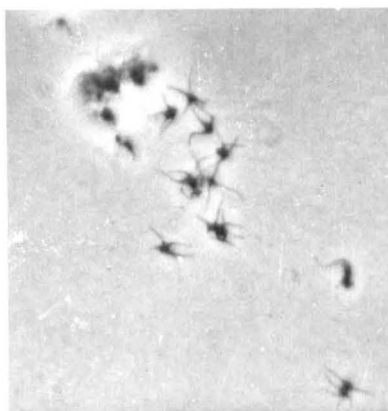


Plate 2.4

Ancalemicrobium species (A)

Phase contrast x 1120.

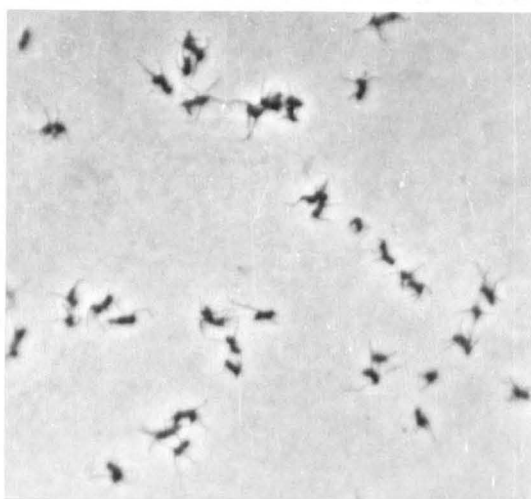


Plate 2.5

Ancalemicrobium species (B)

Phase contrast x 1120.

Ancalemicrobium (B): Growth and reproduction

Unlike Ancalemicrobium (A), isolate (B) could be maintained and cultured on peptone medium containing vitamins (medium P) or on glucose, yeast extract medium (Medium G + Y) and proved amenable to further study. A

gold palladium shadowing in the electron microscope (Plate 2.6,2.7).

Staley (1968) observed many variations of fresh-water appendaged bacteria by direct electron microscopy and assigned morphological variants to different groups. Although the two isolates referred to above show obvious morphological distinctions they will both be referred to as Ancalomicrobium species because of the similar mode of growth, number and formation of appendages.

Ancalomicrobium(A): Growth and reproduction.

The first isolate (A) is morphologically indistinguishable from the Ancalomicrobium species observed by Staley (figure 2.8,1968) and corresponds in growth and reproduction to Ancalomicrobium adetum i.e. reproduction is by budding from one point on the cell body and the appendages are not involved (figure 2.13).

Grown on peptone medium supplemented with a vitamin solution this species produced a light brown pigmented, mucoid raised colony. Difficulty was encountered in subculturing this bacterium. The cells grew infrequently and despite a survey of various carbon and energy sources in conjunction with vitamin, amino acid and growth stimulants, no adequate growth medium or culture conditions were found. Consequently the culture could only be sustained for up to three transfers after initial isolation.

Ancalomicrobium (B): Growth and reproduction

Unlike Ancalomicrobium (A), isolate (B) could be maintained and cultured on peptone medium containing vitamins (medium P) or on glucose, yeast extract medium (Medium G + Y) and proved amenable to further study. A

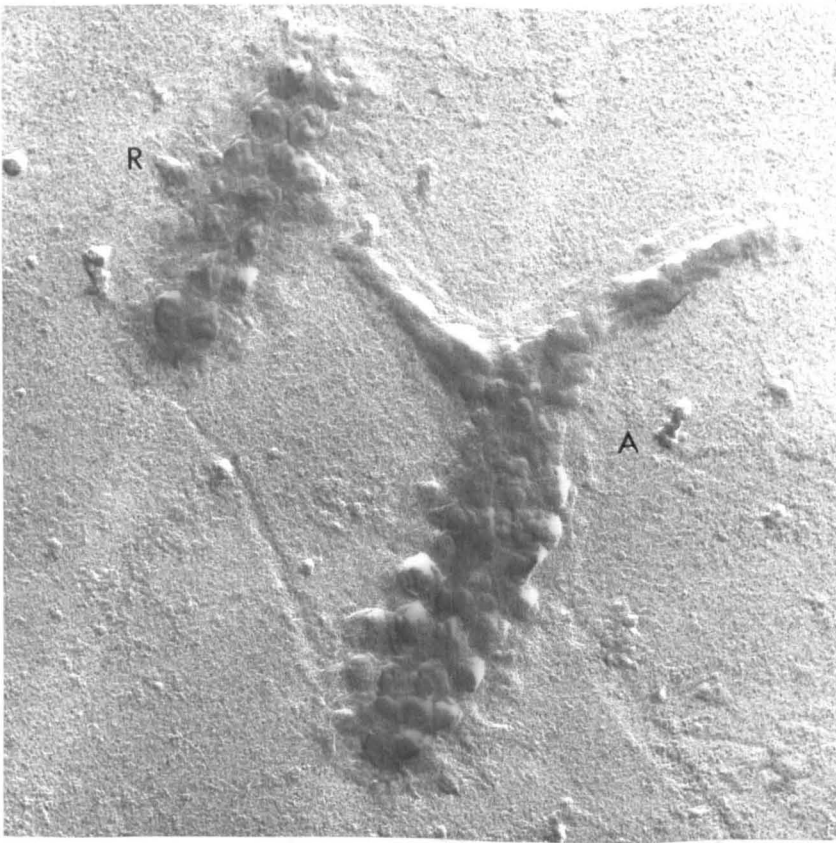


Plate 2.6

Acanthamoeba(B) shadowed with gold palladium
x 25,000.

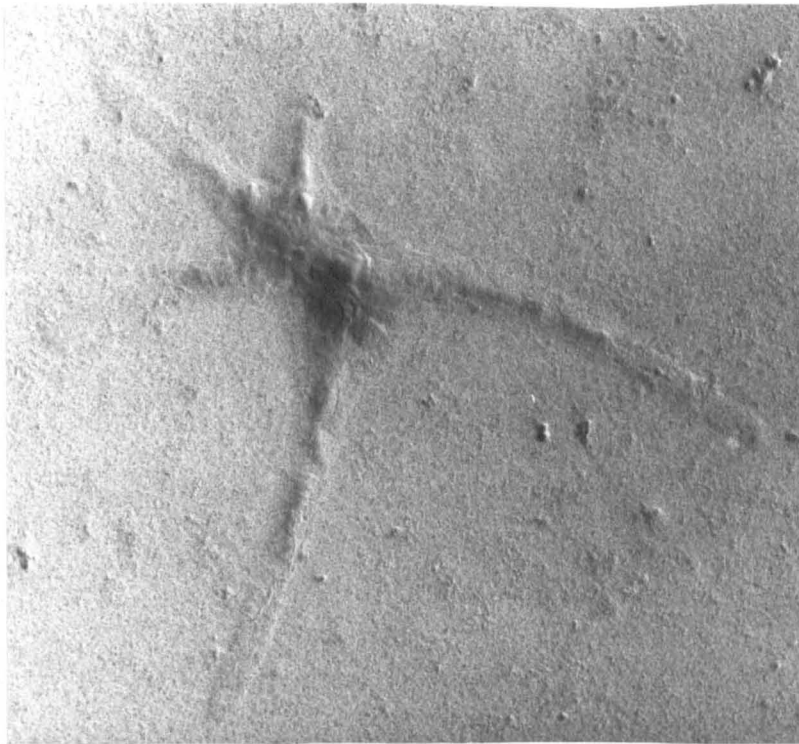


Plate 2.7

Acanthamoeba(A) shadowed with gold palladium
x 25,000.

Note that in both electron micrographs the cytoplasm of the cell proper is continuous with that of the appendages.

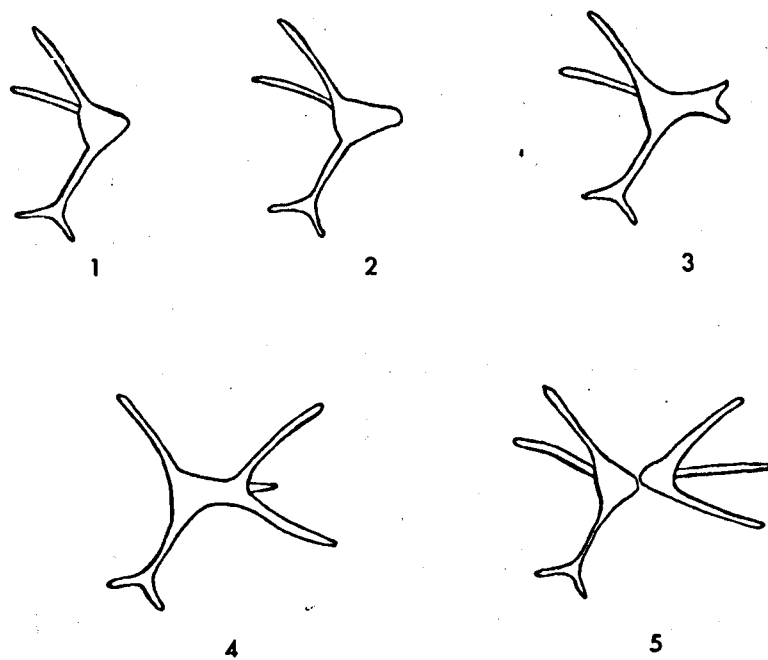


Figure 2.13

Division cycle of Ancalomicrobium species.

(Staley, 1968)

distinctive rod shaped cell, isolate (B), demonstrated a polar mode of growth similar to Ancalomicrobium (A) with appendage induction and maturation on the daughter cell (Plate 2.11, figure 2.14). On either medium (P or G+Y) a raise, mucoid, translucent orange pigmented colony was characteristic of the organism.

Morphological variation.

Loss of appendages and distinctive pleomorphy (y shape) were prevalent when cells were grown on medium containing organic nutrients (glucose, peptone or yeast extract) in excess of 200 µg/ml (Plate 2.8).

The generation time on medium P or G+Y was found to be 6 hrs. whereas on medium containing 200 µg/ml of organic nutrients it was 12-15 hrs. Under these conditions cells remained viable for only one or two transfers and frequently lysed when prepared for observation by phase contrast microscopy suggesting a weakness of wall structure. These findings serve to emphasise the necessity for dilute organic nutrient concentrations in enrichment culture, not only to prevent overgrowth by unwanted bacteria but to permit growth and reproduction, in a recognisable form, of multi-appendaged cells.

When grown in liquid medium and subsequently used to inoculate spread plates, two colony types, in approximately a 1:1 ratio, invariably arose after several days incubation. One colony type was identical to that of the 'parental' clone, the other was similar but with a much more intense colouration. Microscopic examination showed that the intensely orange pigmented colonies contained rod shaped non-appendaged bacteria, whereas the other colonies

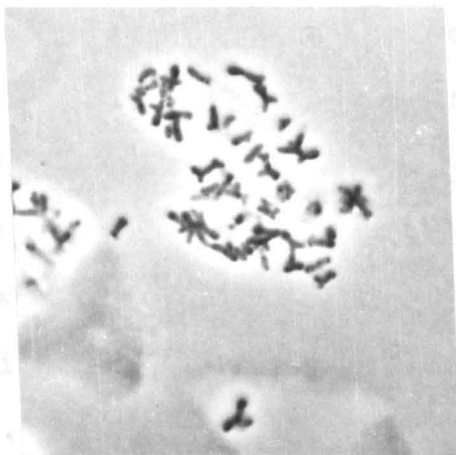
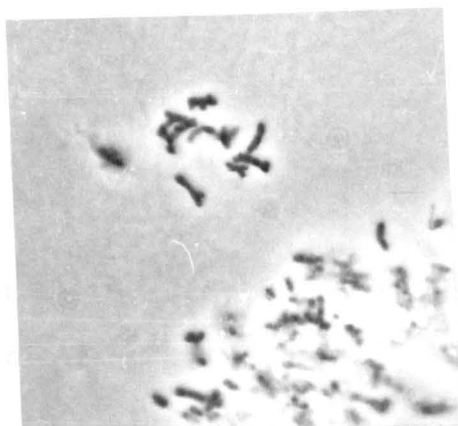
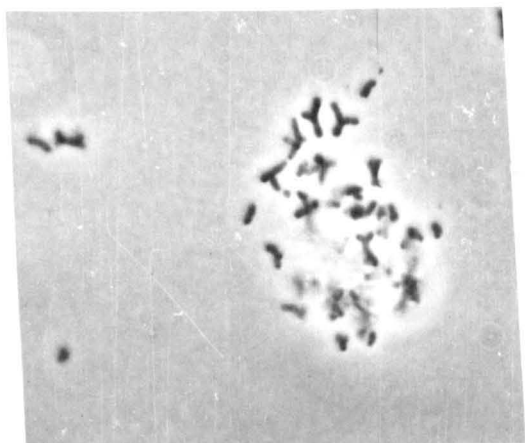


Plate 2.8

Non appendaged, pleomorphic cells of Ancalomicrobium (B) induced by growth on medium containing organic nutrients in excess of 200 ug/ml.

Phase contrast x1120.

were as normal. Recloning of either colony followed by further incubation and growth gave the same results. The possibility of 'carry over' contamination was ruled out by repeated recloning and purification.

Appendage induction and repression.

To investigate the above observations further the possibility of appendage induction and repression being a manifestation of environmental or growth conditions was considered.

Schmidt and Stanier (1966) reported stalk variation in Caulobacter from 20 μ , when nutrients were very dilute, (Plate 2.9) to 3 μ , when nutrient concentrations were high. Further to this they showed that stalk elongation could be induced by limiting the concentration of inorganic phosphate. The filament length of Hyphomicrobium and Rhodomicrobium has been shown to respond in a similar manner (this thesis). Ancalomicrobium (B) was therefore subjected to variation of the inorganic phosphate concentration (Table 2.5, Plate 2.10). From these results it is apparent that appendage length, number and cell morphology can be drastically altered by varying environmental parameters. Appendage production could not be totally repressed under these conditions except when the cells became very pleomorphic and concomitantly non-viable. The question as to the functional factor involved in this system i.e. variation in the phosphate concentration being a unique physiological trigger has been brought into question by continuous culture work on Hyphomicrobium (Harder, pers. comm.). In a steady state system growth rate alone

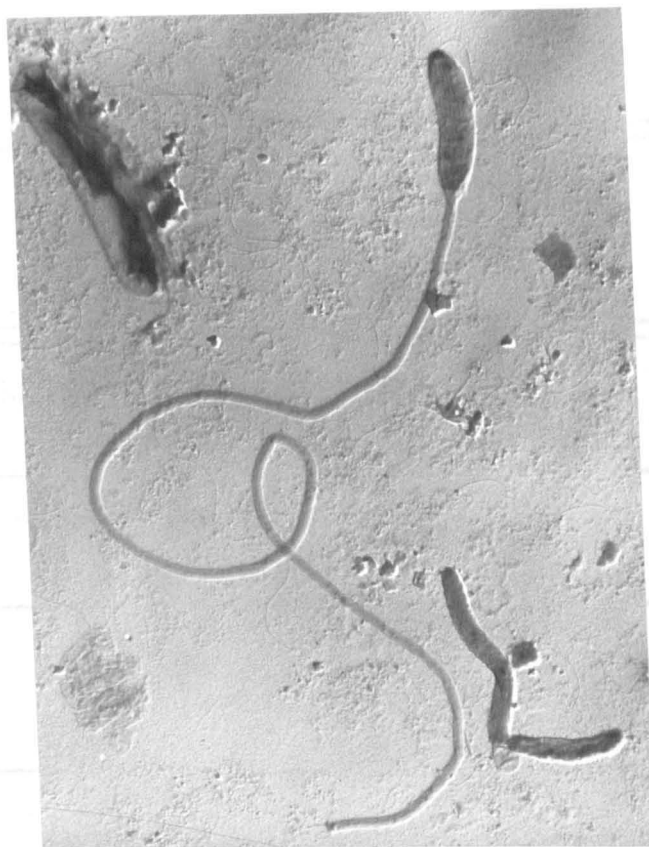


Plate 2.9

Caulobacter spp. growing in a dilute peptone enrichment medium lacking added inorganic phosphate.

Electron micrograph x 6000.

Shadowed with gold palladium at an angle of 15° .

Dimensions of cell body $3 \mu \times 0.5 \mu$.

Stalk length 25μ .

Table 2.5

The effect of phosphate concentration on appendage length, number per cell and on cell morphology of Ancalomicrobium (B) after 24 hrs. incubation.

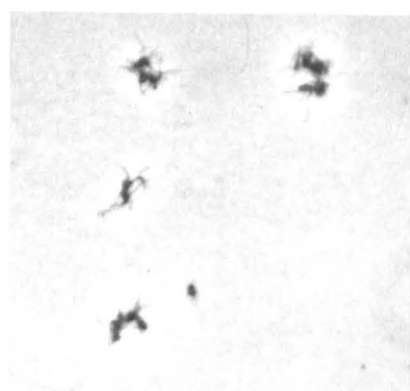
Concentration of inorganic phosphate (molarity)	Stalk length.	No. of appendages per cell.	cell morphology
No added phosphate	3-4 μ	4-5	'normal'
10^{-5}	1-2 μ	8-10	Short, fat rods.
10^{-4}	1-2 μ	8-10	Elongated fat rods.
10^{-3}	0-1 μ	-	Short fat rods.
2×10^{-3}	0-1 μ	-	Pleomorphic y-shaped rods.

Plate 2.10

The effect of phosphate concentration on appendage length, number per cell and on cell morphology of Ancalomicrobium (B) after 24 hours incubation.

1. No added inorganic phosphate.
2. 10^{-5} molar
3. 10^{-4} molar
4. 10^{-3} molar
5. 10^{-3} molar
6. 2×10^{-3} molar
7. 2×10^{-3} molar.

Phase contrast x 1250.



1



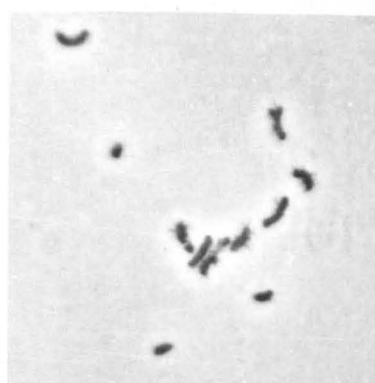
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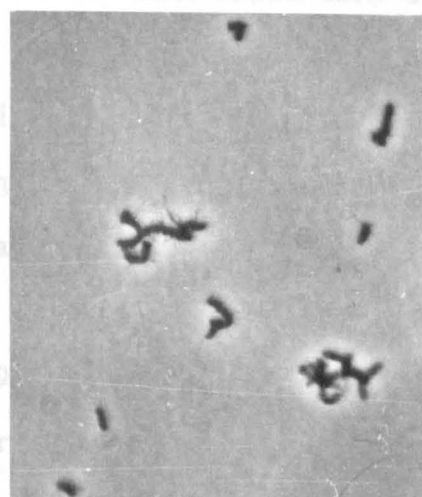
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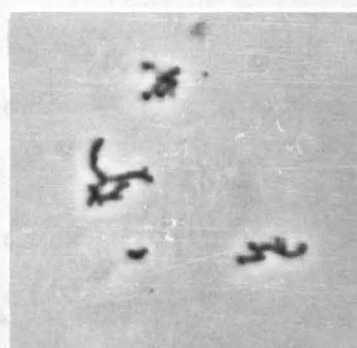
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3



6



7

is responsible for filament length. Similar conclusions may be drawn from slide culture studies on Rhodomicrobium (this thesis). Evidence suggests that in both these systems phosphate limitation is expressed via a modification in the growth rate. These observations lead to the question as to what is the physiological function of the Caulobacter stalk and the Ancalomicrobium appendages? In neither system do they play a reproductive role. Are they, therefore, flotation devices or perhaps uptake 'organelles' capable of being induced or repressed by environmental stimuli? Physiological and ultrastructural evidence supports the latter.

Cohen-Bazire et al (1966) have shown that Caulobacter crescentus contains two types of membranous organelles, one situated at the site of stalk formation and continuous with the membranous core of the stalk is unique to the Caulobacteriales, the other is a mesosome type of structure derived by complex intrusion of the cytoplasmic membrane and functional in division and respiratory metabolism. The principle physiological evidence comes from studies by Schmidt and Samuelson (1972) on the effects of cyclic nucleotide and nucleoside triphosphates on stalk formation in Caulobacter crescentus. Several nucleoside triphosphates have been found to be exogenous effector compounds of inhibition of stalk synthesis whereas the reciprocal effect, enhancement of stalk elongation, is produced by the addition of cyclic GMP. These are the subcellular regulatory triggers, indirectly controlled by the environment.

Such studies on Ancalomicrobium (B) were hindered by the difficulties encountered in growing this micro-

organism on a defined medium. Staley (1968) however, published ultrastructural electron micrographs of Ancalomicrobium adetum. No organelle corresponding to that found in Caulobacter is present. It may therefore be pertinent to suggest that the Caulobacter stalk has evolved to a high degree of efficiency i.e. become a highly organised uptake system, whereas the appendages of Ancalomicrobium lack such sophistication. Consequently, to attain similar uptake capabilities, Ancalomicrobium surmounts the problem by possessing several inducible appendages. The question of the physiological relationship between the filaments of Hyphomicrobium and Rhodomicrobium and the appendages of Caulobacter and Ancalomicrobium remains unresolved.

Wall structure.

The unique and distinctive regular subunit array of the cell wall of Ancalomicrobium (B) (Plate 2.11,2.13) under normal cultural conditions suggests that appendage production is synonymous in origin with the protruberances, i.e. cell wall structure is analogous with appendage production. When grown in high concentrations of organic nutrients the regular wall structure is lost, although irregularities persist (Plate 2.14,2.15) and the cell becomes osmotically fragile, as judged by the tendency to lyse and the appearance of sphaeroplasts (Plate 2.14).

Motile cells

A further anomaly found with this organism is the infrequent and inconsistent presence in liquid cultures of motile non-appendaged, ovoid cells which ultimately form the regular subunit structure of the mature cell (Plate 2.13) and become appendaged. The factor(s) influencing or

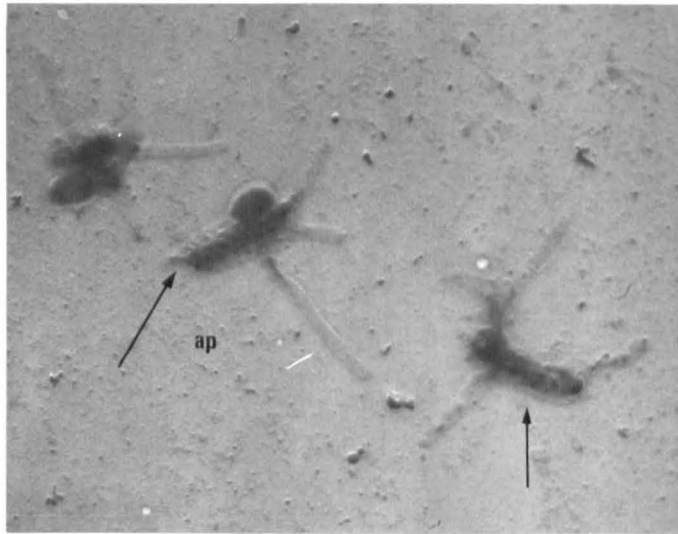


Plate 2.11

Polarly appendaged cells showing polar growth, initiation and development of appendage synthesis on derivative daughter cells. Electron micrograph of a gold palladium shadowed preparation of Ancalomicrobium(B) x6000.

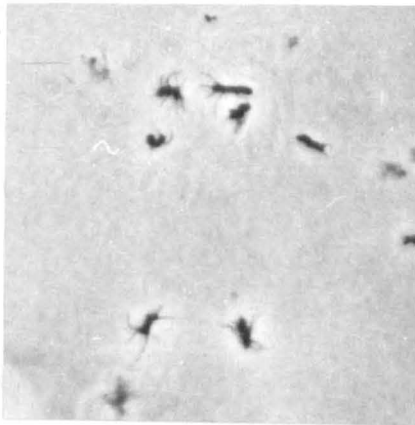


Plate 2.12

Ancalomicrobium (B)
Phase contrast x1120.

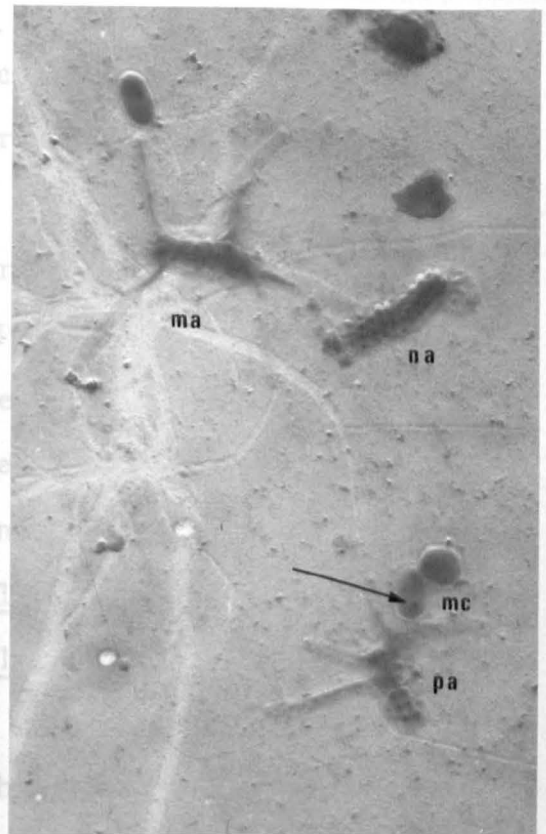


Plate 2.13

Electron micrograph of a gold palladium shadowed preparation of Ancalomicrobium(B) x6000.

ma Mature appendaged cell prior to division.
pa Polarly appendaged cell.
na Non appendaged cell showing regular array of subunit structure
mc Motile, ovoid, non appendaged cell
Initiation of subunit structure
arrowed

determining their appearance in a culture or their formation were not conclusively determined. However, from the evidence available (electron micrographs) it appeared as if they were derived from rod shaped non-appendaged cells by polar growth.

There is not an obligate differential life cycle e.g. as in Caulobacter, but, a situation similar to that experienced with Geodermatophilus (Ishiguro and Wolfe, 1970) where there are complex morphological variations in response to environmental stimuli. The information revealed during this study on the growth and reproduction of Ancalomicrobium(B) is summarised in figure 2.14.

Literature anomalies.

The following organisms have been described during the course of direct electron microscopic investigations of various soil and peat samples (Stefanov and Nikitin, 1965; Orenski, 1966 a and b; Volarovich and Terent'ev, 1968).

Helicoidal polyspheroides (Plate 2.16) has been characterised as a rod, 0.5 μ wide and one to seven μ long, covered with rounded protruberances 0.1 μ in diameter and arranged in helical rows (Orenski, 1966, a and b). A similar organism Agrobacterium polyspheroidum (Plate 2.17) has been observed by Nikitin et al (1966). Tuberoidobacter, (Plate 2.18) another of Nikitin's soil microorganisms, is a Gram negative rod with many "tubercular appendages, about 0.1 μ wide and of varying lengths, extending in all directions from the cell".

In light of the morphological variation of Ancalomicrobium (B), the variation in the number, or even complete absence of appendages and the distinctive wall

Figure 2.14

Growth and development of Ancalomicrobium (B)
summarised diagrammatically.

- AC: Appendaged cell cycle induced in medium containing organic nutrients at a concentration of 100 µg/ml.
1. Polar growth.
 2. Daughter cell appendage formation.
 3. Division.
- NAC: Non appendaged cell cycle in medium containing organic nutrients in excess of 175 µg/ml.
- | | | |
|---|--------|---|
| $\left. \begin{array}{l} 1. \\ 2. \\ 3. \end{array} \right\}$ | growth | followed by 'normal'
binary fission. |
|---|--------|---|
- MC: Motile cell cycle. Stimulatory factors were undetermined.
1. Motile, ovoid cell having no subunit wall structure.
 2. Initiation of subunit structure.
 3. 'Maturation'.

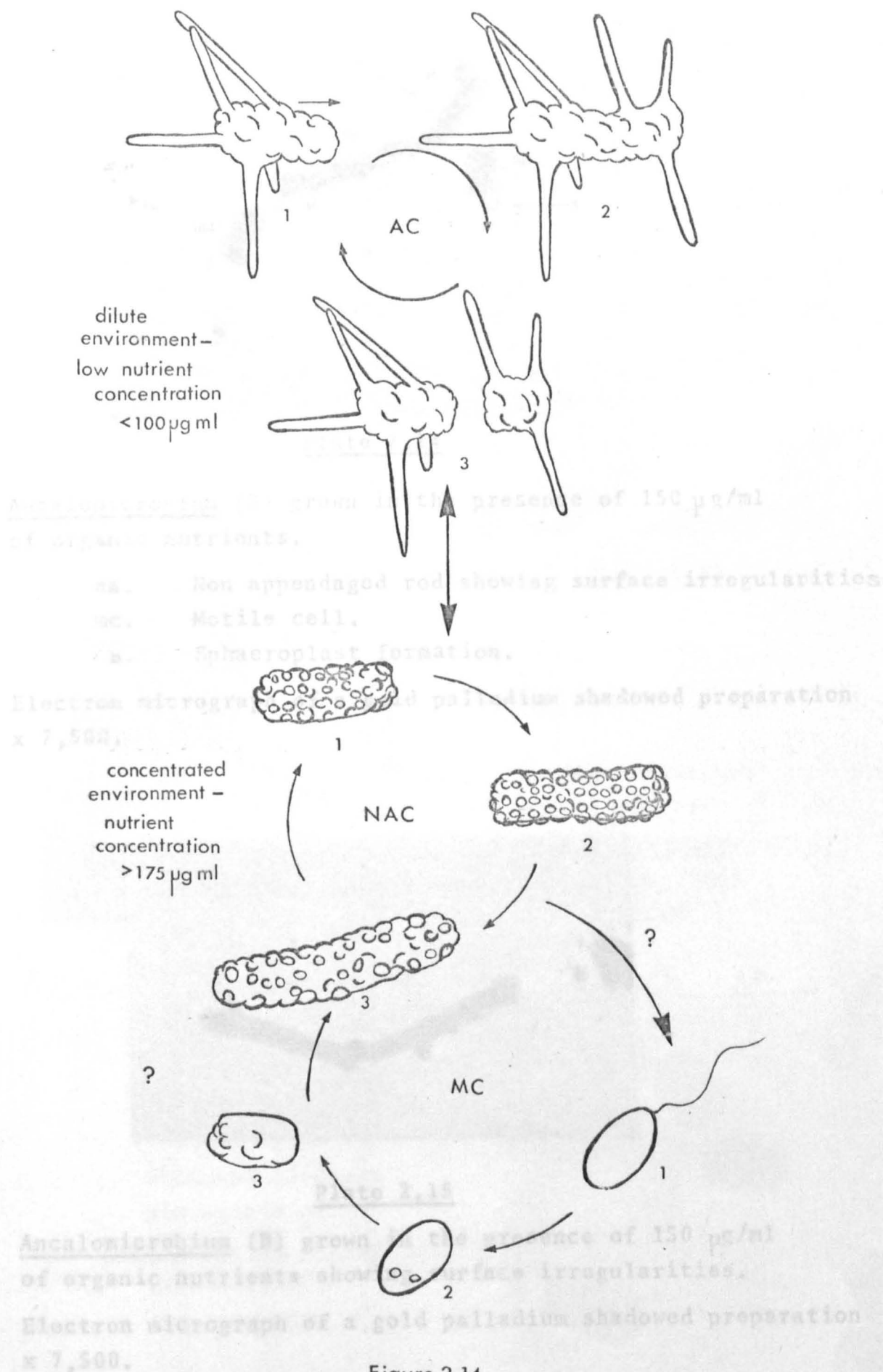


Figure 2.14



Plate 2.14

Ancalomicrobium (B) grown in the presence of 150 $\mu\text{g/ml}$ of organic nutrients.

na. Non appendaged rod showing surface irregularities.

mc. Motile cell.

s. Sphaeroplast formation.

Electron micrograph of a gold palladium shadowed preparation x 7,500.

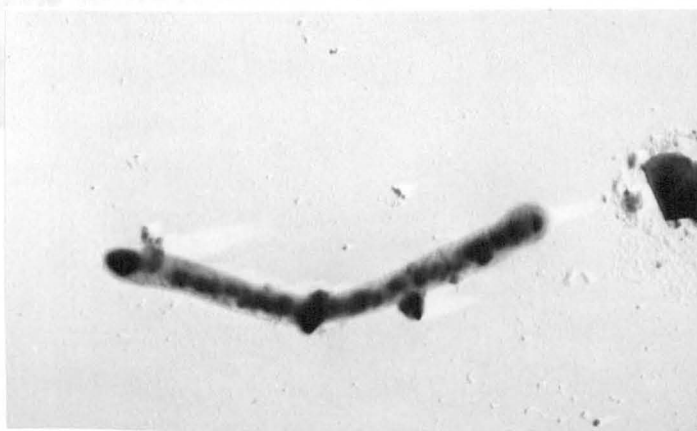


Plate 2.15

Ancalomicrobium (B) grown in the presence of 150 $\mu\text{g/ml}$ of organic nutrients showing surface irregularities.

Electron micrograph of a gold palladium shadowed preparation x 7,500.

Shadowed electron
micrograph of
Helicoidal polyspheroides
x 16000.

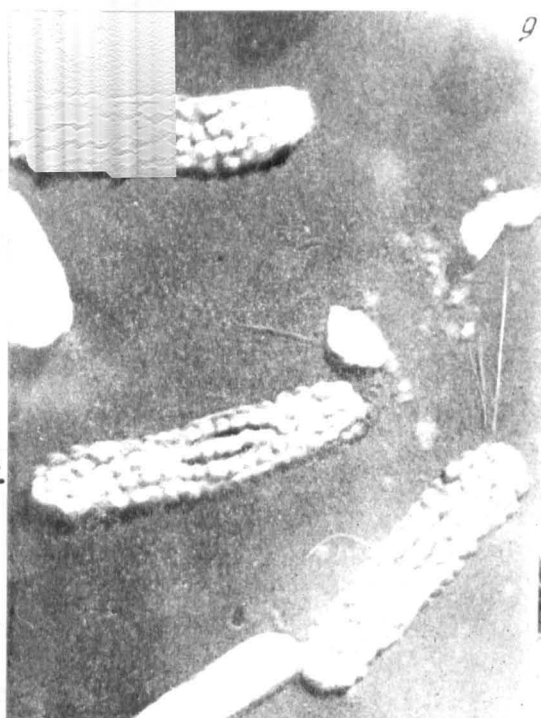
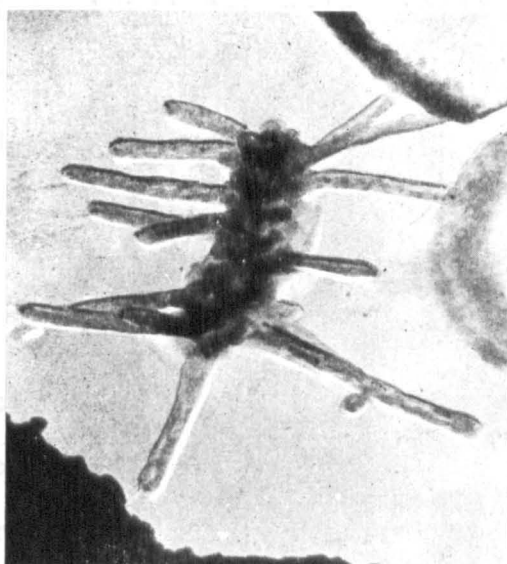


Plate 2.16



Shadowed electron
micrograph of
Tuberoideobacter.
(Nikitin, 1966)
x 19000.

Plate 2.17

Shadowed electron
micrograph of
Agrobacterium polyspheroidum
(Nikitin, 1973)

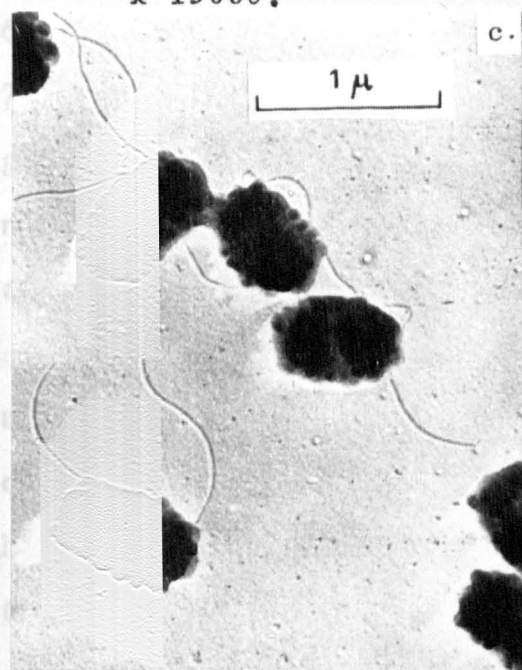


Plate 2.18

structure casts a shadow on the validity of the aforementioned genera especially since the bulk of the evidence so far reported is based on electron micrographs of 'natural' samples and not on cultured isolates. These genera can be identified simply as morphological stages of the expression of Ancalomicrobium (B) or a related species.

Hyphomicrobium - Morphological variation.

Hyphomicrobium is usually seen as a pear-shaped cell reproducing by the production of a bud at the end of a long, unbranched filament (Kingma Boltjes, 1936; Mevius, 1953; Zavarzin, 1961). The resultant flagellated swimmers or daughter cells ultimately lose their flagella, produce filaments and repeat the process while the mother cells produce further buds on the same filaments (Bauld and Tyler, 1971). Variation from this classical morphology has been reported (Zavarzin, 1961; Hirsch and Conti, 1964b; Tyler and Marshall, 1967b; Bauld and Tyler, 1971; Bauld, Tyler and Marshall, 1971) and has given rise to controversy over the validity of Aristovskaya's (1961) genus Pedomicrobium (Conti and Hirsch, 1964b; Hirsch, 1968; Tyler and Marshall, 1967b; Bauld, Tyler and Marshall, 1971; Bauld and Tyler, 1971) with the evidence strongly supporting a recommendation that this genus be abolished and signalling caution in the adoption of morphological features and variations as genetic criteria.

The interest shown in the pleomorphy of Hyphomicrobium has arisen in part from the ability of members of this genus to deposit iron and manganese from the environment (Hirsch, 1968; Tyler and Marshall, 1967a, 1970).

Metal oxide deposition.

The strains of Hyphomicrobium isolated during this study were found to be capable of depositing manganese but not, as far as could be determined, as a consistent response to the environment, i.e. medium composition or cultural conditions, although once initiated deposition was prolific (Plate 2.19, 2.20, 2.21). Similar observations have been made on the epicellular deposition of ferric hydroxide (Hirsch, 1968).

In an attempt to explain these observations, Hirsch (1968) conceived that the initial oxidation and deposition is caused by local change of cell surface pH through microbial action e.g. by the excretion of NH_3 during autolytic processes or by the presence of a specific catalytic surface material on the wall, so giving rise to 'primary active sites'. Further "growth" of the deposit could then be a consequence of non-microbiological oxidation and deposition.

Careful study of negatively stained electron micrographs of Hyphomicrobium cells reveals an abundance of cell wall 'catalytic', i.e. primary active sites, (Plates 2.19, 2.25) bearing comparison with those detailed by Hirsch (1968) and attributed to the genus Pedomicrobium.

Morphological variation.

Plates 2.22 and 2.23 show the classical reproduction and growth in the exponential phase of Hyphomicrobium grown aerobically with methanol as a sole carbon and energy source. Two strains, however, Hyphomicrobium X and Hyphomicrobium C (obtained from Dr. W. Harder, Univ. of Groningen) when grown in the presence of methanol and nitrate under a

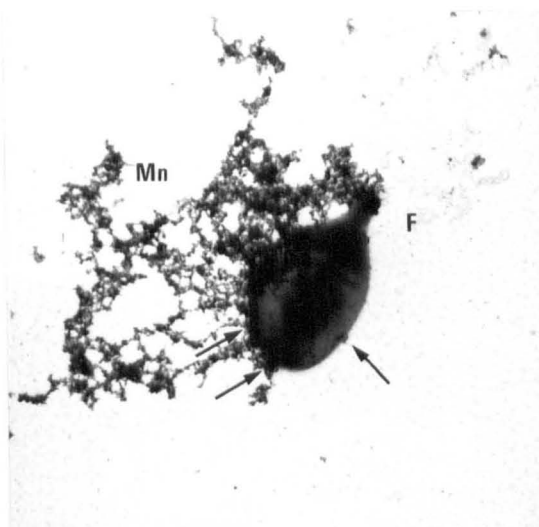
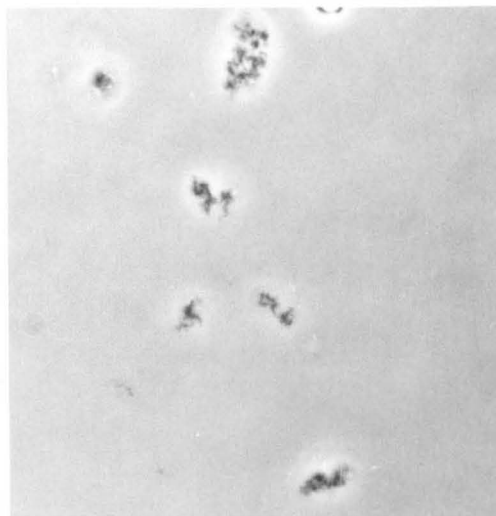
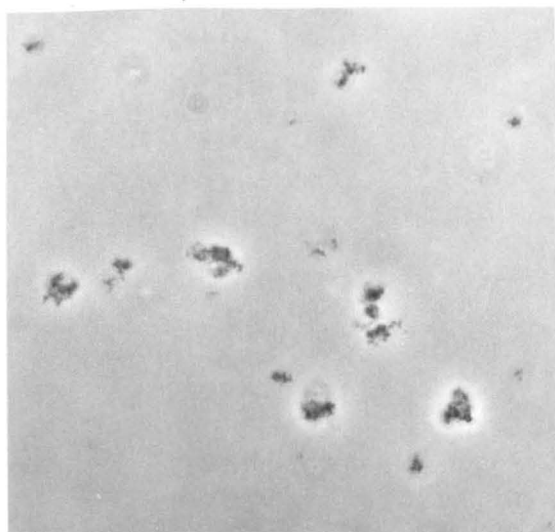


Plate 2.19

Negatively stained (phosphotungstic acid) electron micrograph of a developing Hyphomicrobium cell showing manganese deposition (Mn) and 'primary active sites'. (x 18000)

F. Filament formation



Plates 2.20, 2.21

Phase contrast photomicrographs of heavy manganese deposition by Hyphomicrobium.

x 1120

Plate 2.22

Gold palladium shadowed electron micrograph
of Hyphomicrobium c.

(M) Mother and developing daughter cell (D).

(F) Filament (x 18,000)

Plate 2.23

Hyphomicrobium c grown on methanol (2 days
incubation).

1. Non stalked swarm cell.
2. Tube development.
3. Bud formation.
4. Bud maturation.

Phase contrast x 1120.

Plate 2.24

Hyphomicrobium c grown on methanol (7-8 days
incubation).

appearance of trilobed cells.

Phase contrast x 1120.

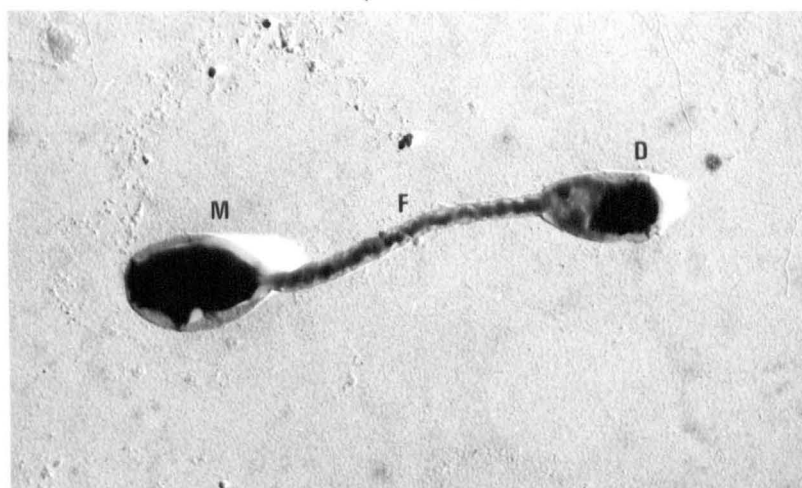


Plate 2.22

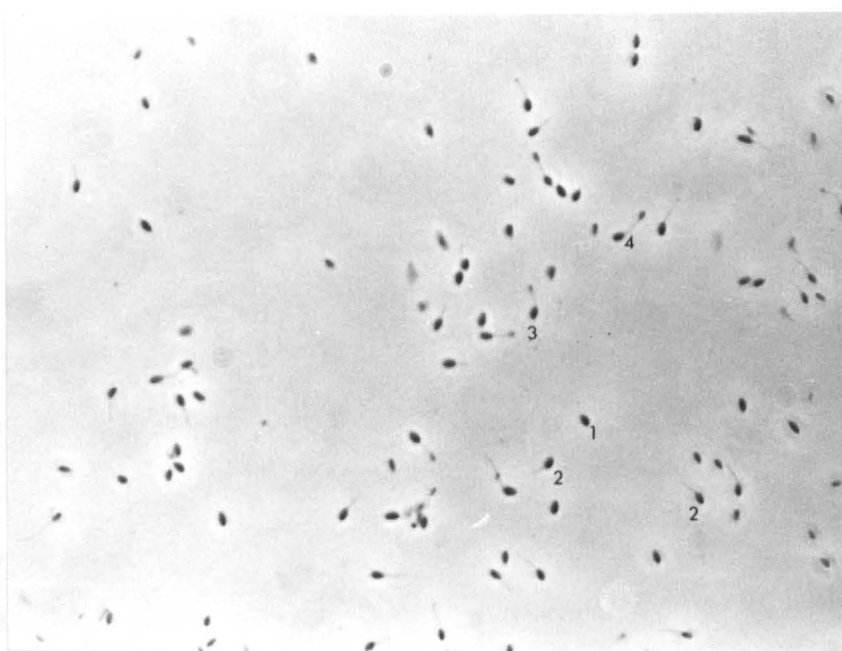


Plate 2.23

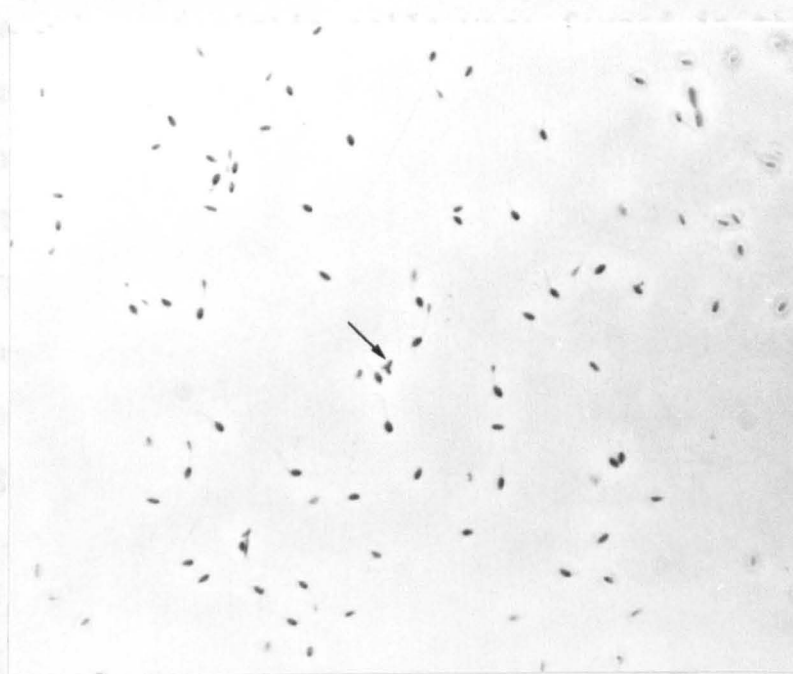


Plate 2.24

reduced partial pressure of O_2 were not only characterised by this morphology but exhibited cellular pleomorphy in the form of dichotomous lobing (Plates 2.24, 2.25, 2.26, 2.27).

Initially it was conceived that these cellular structures might be resting or resistant entities synonymous with the resistant cell characteristic of several Rhodomicrobium isolates (Gorlenko, 1969; this thesis, section 3.3). Further characterisation disproved this, i.e. no detectable cellular resistance could be demonstrated (heat, UV, or chemical).

In Hyphomicrobia, cell shape, length of hyphae and degree of branching depends on cultural conditions (Hirsch and Conti, 1964b; Tyler and Marshall, 1967b; Bauld, Tyler and Marshall, 1971). Further to these observations Bauld, Tyler and Marshall (1971) demonstrated that pleomorphy in Hyphomicrobium (T37) could be manipulated specifically by the use of two C_1 carbon sources, methanol and methylamine. Their investigations have been, in part, confirmed and substantiated in the work reported here.

Trilobed cell formation.

These characteristic cells were formed in the late exponential growth phase of methanol grown cultures by budding from the filament tip of morphologically 'normal' mother cells, i.e. similar to 'normal' daughter cell formation. The initial sequences were identical to flagellated cell production, morphological variation only being observed late in formation. Subsequent development was by enhancement of the trilobed appearance prior to division and cell release (figure 2.15, Plates 2.28, 2.29, 2.30).

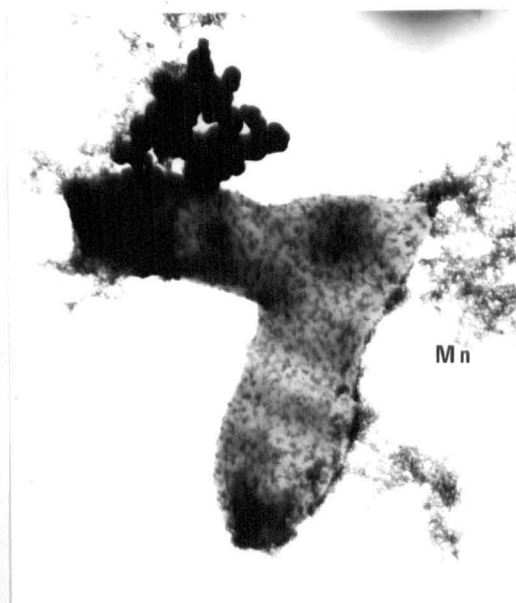
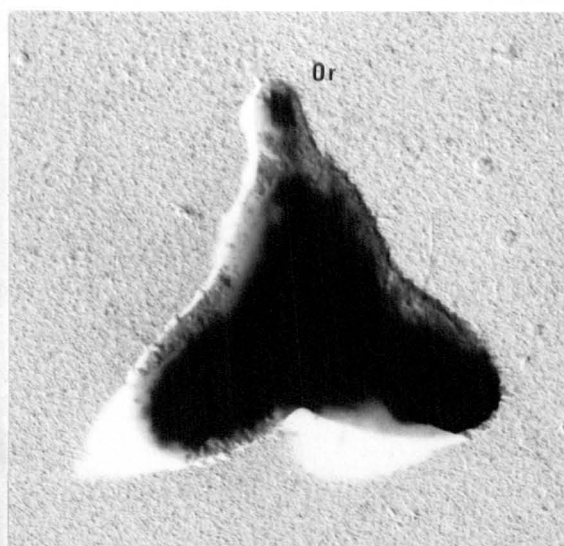
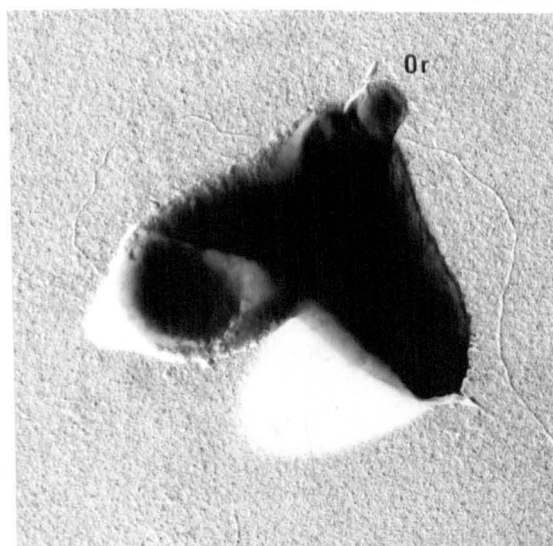


Plate 2.25

Negatively stained (phosphotungstic acid) electron micrograph of trilobed cell formed in a methanol grown culture of Hyphomicrobium c after 7 days incubation. (Note manganese deposition (Mn) and 'primary active sites') (x 36000)

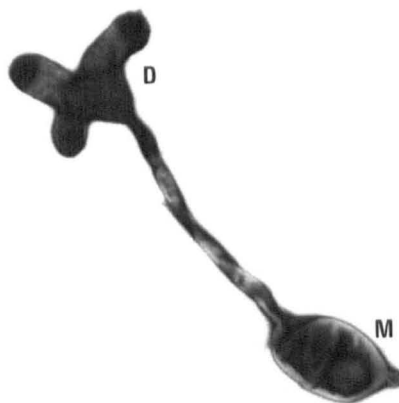


Plates 2.26, 2.27

Gold palladium shadowed electron micrographs of trilobed cells of Hyphomicrobium c. (x 36000)

Or - Point of origin from mother cell.

(x 25000)



Plates 2.28, 2.29

Negatively stained (phosphotungstic acid) electron micrographs of trilobed cell formation from 'normal' mother cells (M), showing initial and subsequent dichotomous growth of the daughter (D).

(x 15000)



Plate 2.30

Gold palladium shadowed electron micrograph of trilobed cell formation in Hyphomicrobium c grown on methanol.

x (x 25000)

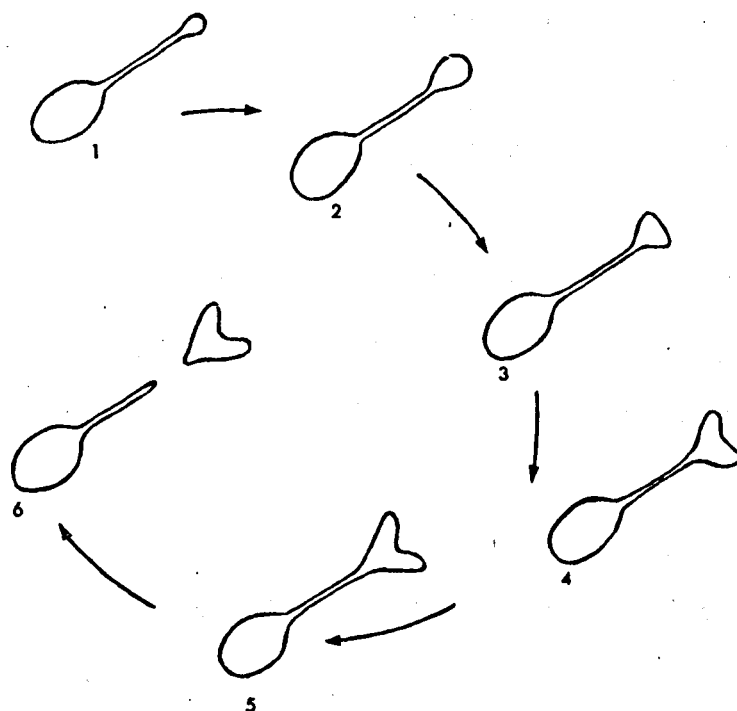


Figure 2.15

Trilobed cell formation from 'normal' mother cell found to occur during late exponential phase of methanol grown cultures. Diagrammatic representation of events as followed in liquid culture by phase contrast microscopy.

1. Bud formation.
2. Bud development.
3. Bud assumes triangular morphology.
4. Dichotomous branching.
5. Branching accentuated.
6. Division.

Trilobed cell growth and development.

When trilobed cells appeared in a culture they were seldom in large numbers but often solitary or as buds from mother cells having failed to separate. Subsequent development was found to follow one of the following:

- a) When given additional methanol during the late exponential growth phase (50mM) trilobed cells free in the medium developed a classical filament (Plates 2.31,2.32) followed by normal motile cell formation. This may be indicative that pleomorphy is a response to a physiological (metabolic) shift brought about by specific C_1 starvation. Under cultural conditions where the concentration of added methanol was severely limited (5mM) trilobed cells gave rise to normal filament formation but cell development resulted in a daughter trilobed cell (Plate 2.33).
- b) The second mode of growth can be described as repeated dichotomous lobing followed by bud formation (Plates 2.34, 2.35,2.36) and is induced by alteration of the carbon and energy source, in this instance from methanol (10mM) to methylamine (10mM). Continued growth in methylamine gave rise to irregular colonial formation which lacked recognizable Hyphomicrobium characteristics but closely resembled the bizarre cell shapes of Aristovskaya's Pedomicrobium(1961).

These morphological changes, although extreme, are not randomly pleomorphic but organised and specific. Careful consideration of Plate 2.36 illustrates this point. When followed by phase contrast microscopy over several hours incubation, the sequential events leading to the formation of such a cellular structure are as shown in figure 2.16. The unique growth characteristic of this cell

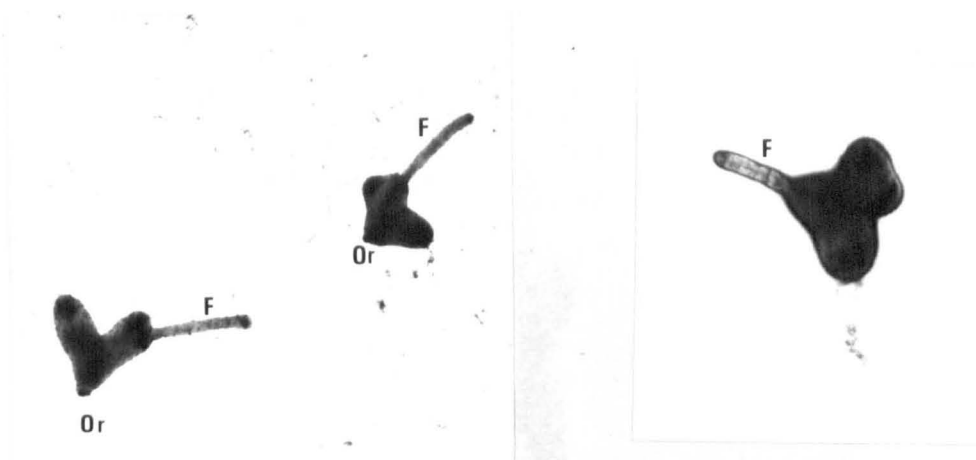


Plate 2.31

Filament (F) formation from trilobed cells given additional methanol in the late exponential growth phase.

Or - Point of origin from mother cell.

Negatively stained (phosphotungstic acid)(x 15000)

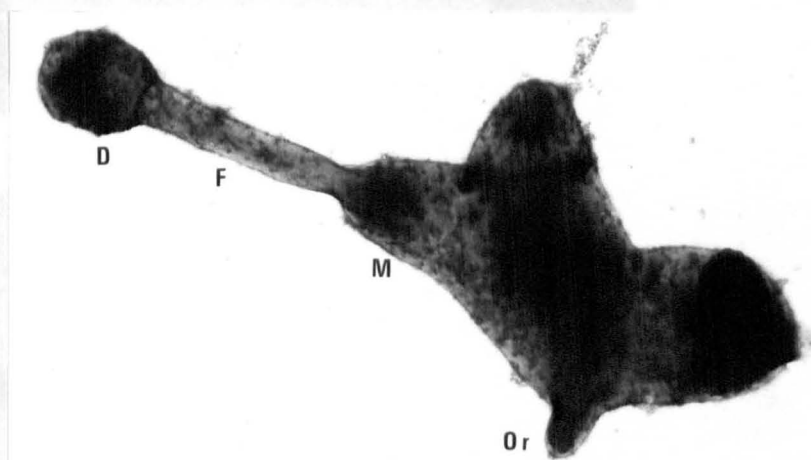


Plate 2.32

Filament (F) and daughter cell formation (D) from trilobed mother cell (M).

Or - Point of origin from mother cell.

Negatively stained (phosphotungstic acid)(x 25000)

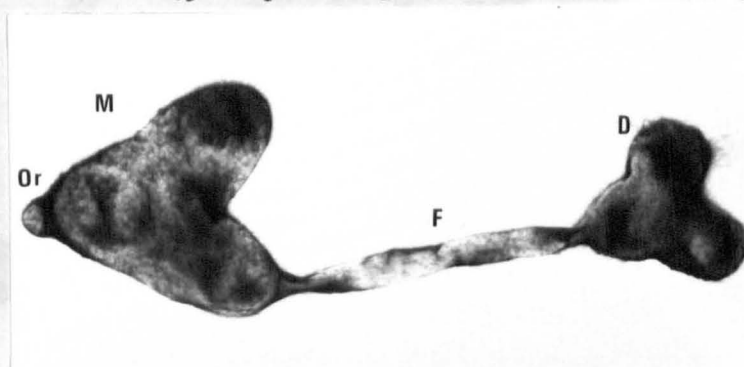


Plate 2.33

Trilobed mother cell (M) giving rise, via normal filament (F) formation, to a trilobed daughter cell (D) under conditions of methanol limitation.

Negatively stained (phosphotungstic acid)(x 25000)



Repeated dichotomous growth followed by bud (5)
 anthesis.



1d) (x 22000)

Plates 2.34, 2.35

Repeated dichotomous lobing induced by carbon source
 variation (methanol to methylamine).

Gold palladium shadow(x 25000)

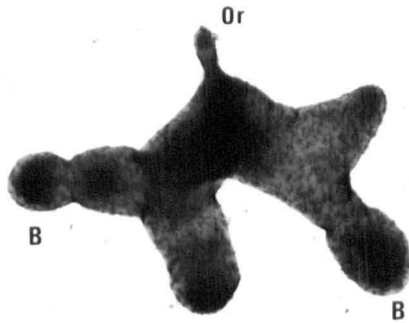


Plate 2.36

Repeated dichotomous growth followed by bud (B) formation without 'normal' filament synthesis.

Or - Point of origin from mother cell.

Negatively stained (phosphotungstic acid)(x 22000)

Dichotomous branching and bud formation of a trilobed cell grown in methylamine.

1. Trilobed cell (as formed in methanol culture)
 2. Initiation of dichotomous branching
 3. Dichotomous branching
 4. Bud (b) and dichotomous arm (d) formation
- Or. Point of origin from mother cell.

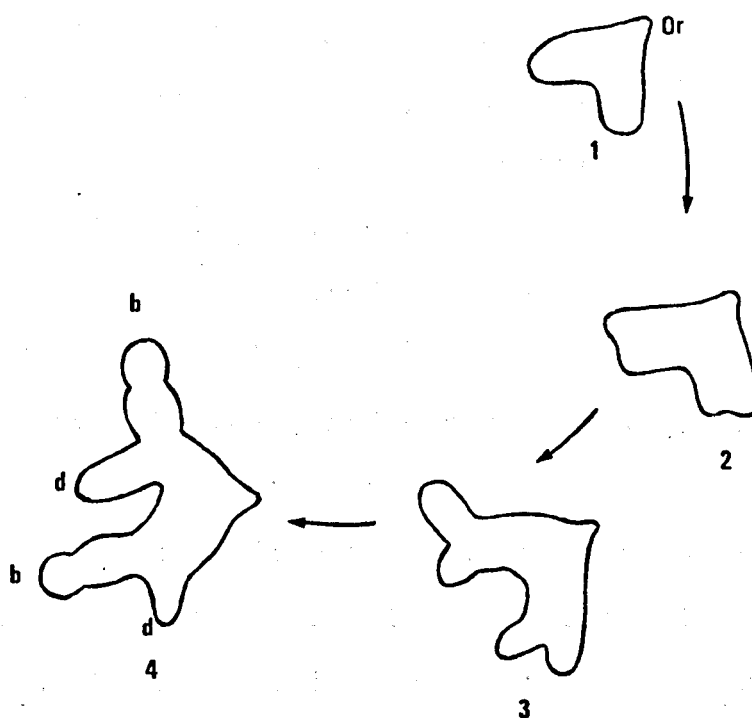


Figure 2.16

Dichotomous branching and bud formation of a trilobed cell grown in methylamine.

1. Trilobed cell (as formed in methanol culture)
 2. Initiation of dichotomous branching
 3. Dichotomous branching
 4. Bud (b) and dichotomous arm (d) formation
- Or. Point of origin from mother cell.

is the dichotomous branching with subsequent division into a 'budding arm' (b) and one destined to produce a second dichotomous branch (d), a feature not readily demonstrable in other procaryotic genera.

Figure 2.17 summarises the morphological adaptations of Hyphomicrobium associated with variation in carbon source and concentration.

The physiological, and environmental consequences of trilobal cell formation remain undetermined. The important conclusion from these tentative observations is that environmental parameters can control phenotypic expression. Therefore, in view of the considerable pleomorphy described, caution must be excersised in erecting new genera of budding bacteria.

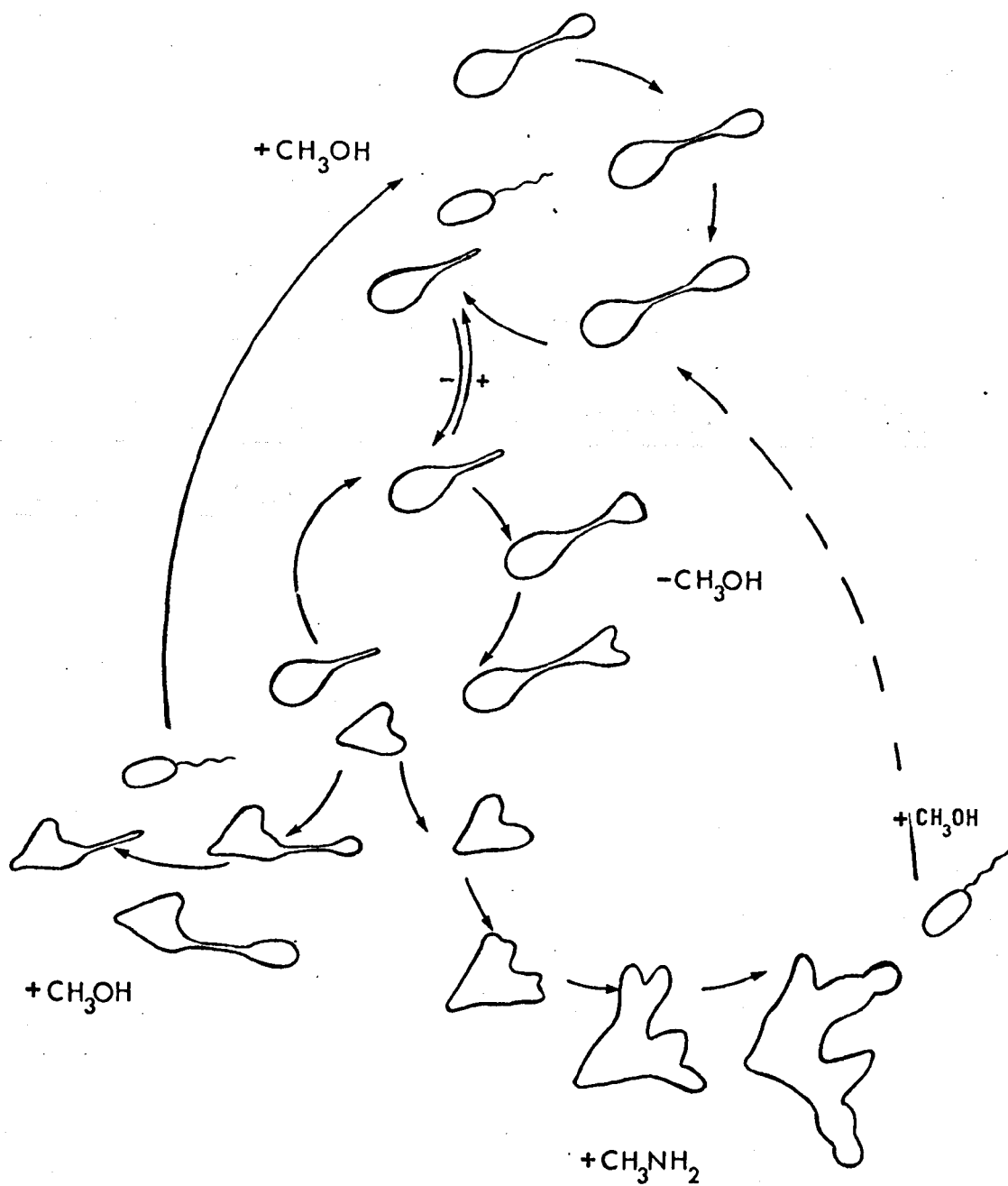


Figure 2.17

Summation of morphological events occurring in *Hyphomicrobium* (strains X or C) in response to variation of carbon source and concentration.

Section 3.

The photosynthetic prosthecae bacteria:

Rhodopseudomonas acidophila, Rh. palustris, Rh. viridis
and Rhodospirillum rubrum.

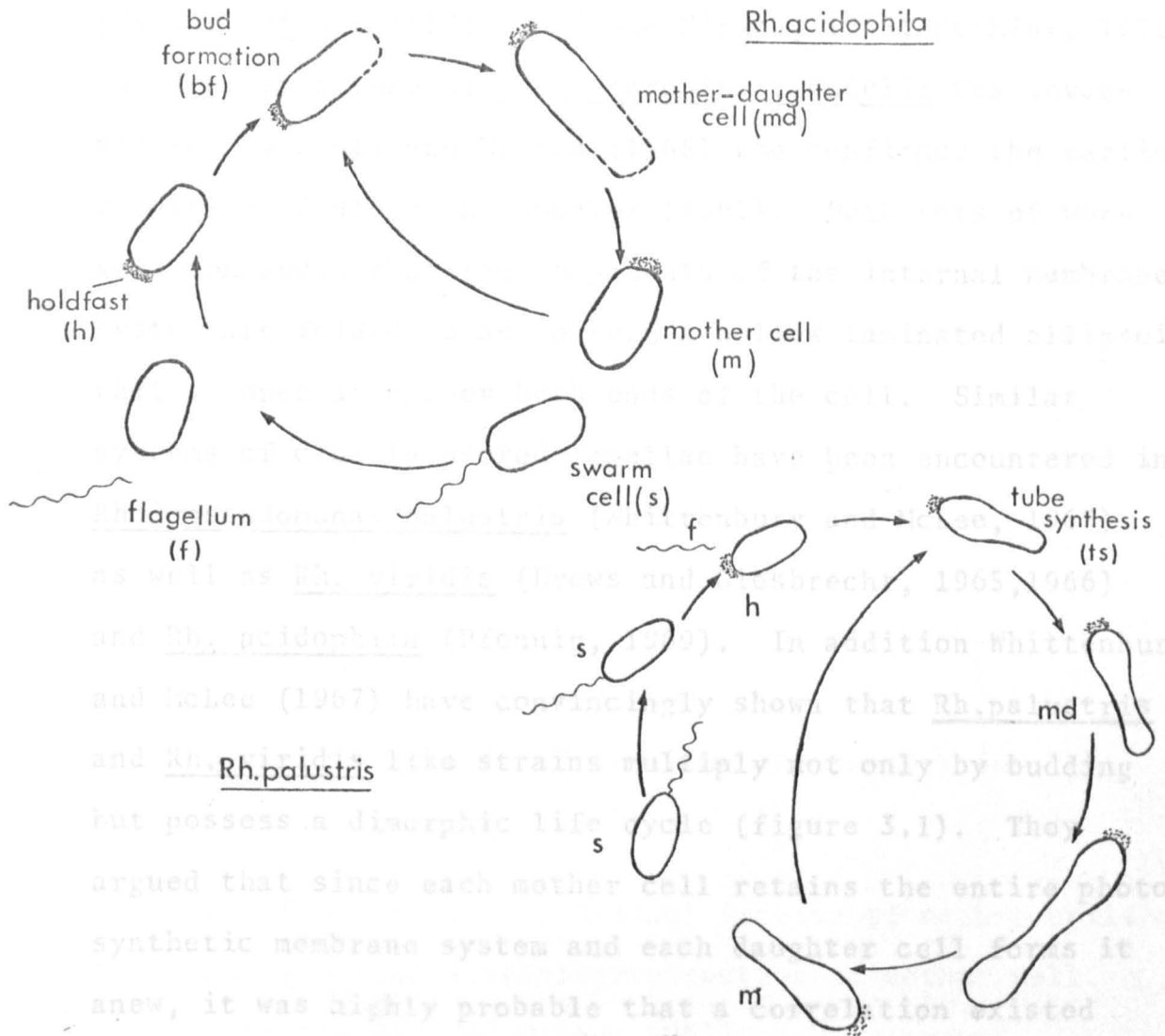
Section 3.1Introduction.

No comprehensive review exists on the budding filamentous bacteria Rhodomicrobium vannielii, Rhodospseudomonas palustris, Rh. viridis and Rh. acidophila out with brief résumés given in composite works on photosynthetic (Pfennig, 1967) and prosthecate procaryotes (Schmidt, 1971; Staley, 1973).

Rhodomicrobium vannielii although first isolated in 1949 by Duchow and Douglas (1949) has subsequently received only brief and intermittent attention mostly in comparative studies with the better documented Hyphomicrobium species (Conti and Hirsch, 1965). The other bacteria of this group have only recently been characterised (physiologically and morphologically), Rhodospseudomonas palustris and Rh. viridis by Whittenbury and McLee (1967) and Rh. acidophila by Pfennig (1969). Comparatively little, however, is known about filament formation, the budding mode of replication, polymorphic life cycles, or holdfast formation. This also applies to budding filamentous non-photosynthetic bacteria.

Rm. vannielii, Rh. palustris, Rh. viridis and Rh. acidophila all occur commonly in freshwater (Hirsch and Rheinheimer, 1968; this thesis) and have with the exception of Rh. viridis (Drews and Giesbrecht, 1966) the general physiological properties associated with the Athiorhodaceae. Their mode of reproduction, however, bears a strong resemblance to that of Hyphomicrobium in that daughter cells, which undergo an obligate differentiation process, are formed by budding (figure 3.1).

Although physiologically affiliated with the Athiorhodaceae Rhodomicrobium occupies a unique taxonomic position due not only to its complex mode of replication but to the

RhodomicrobiumRh. acidophila

argued that since each mother cell retains the entire photosynthetic membrane system and each daughter cell forms it anew, it was highly probable that a correlation existed between the structural feature of a lamellar membrane system, which is extended parallel to the long axis of the cell, and a budding type of replication i.e. the budding process is seen as a mechanism that avoids complex reorganisation of the procaryotic cellular structure (figure 3.2).

In recent reviews (Skerman, 1977; Schmidt, 1971) it is argued that budding bacteria are clearly distinguished from other bacteria in that they have evolved a specialised system of polar growth i.e. a mother cell produces a tube of variable dimensions at the end of which can be seen the developing daughter cell. Whittamby and McLee (1967) suggested the complex lamellar membrane system in relation to a budding mode of growth and proposed that budding be

Figure 3.1

photosynthetic apparatus and the constituent photopigments (Vatter, et al, 1959; Conti and Hirsch, 1965; Schmidt, 1971). The fine structure of Rhodomicrobium vannielii was investigated by Conti and Hirsch (1965) who confirmed the earlier results of Boatman and Douglas (1961). Both sets of workers concluded that the components of the internal membrane system are folded so as to form a hollow laminated ellipsoid that is open at one or both ends of the cell. Similar systems of closely packed lamellae have been encountered in Rhodopseudomonas palustris (Whittenbury and McLee, 1967) as well as Rh. viridis (Drews and Giesbrecht, 1965, 1966) and Rh. acidophila (Pfennig, 1969). In addition Whittenbury and McLee (1967) have convincingly shown that Rh. palustris and Rh. viridis like strains multiply not only by budding but possess a dimorphic life cycle (figure 3.1). They argued that since each mother cell retains the entire photosynthetic membrane system and each daughter cell forms it anew, it was highly probable that a correlation existed between the structural feature of a lamellar membrane system, which is extended parallel to the long axis of the cell, and a budding type of replication i.e. the budding process is seen as a mechanism that avoids complex reorganisation of the procaryotic cellular structure (figure 3.2).

In recent reviews (Skerman, 1967; Schmidt, 1971) it is argued that budding bacteria are clearly distinguished from other bacteria in that they have evolved a specialised system of polar growth i.e. a new cell produces a tube of variable dimensions at the end of which can be seen the developing daughter cell. Whittenbury and McLee (1967) discussed the complex lamellar membrane system in relation to a budding mode of growth and proposed that budding be

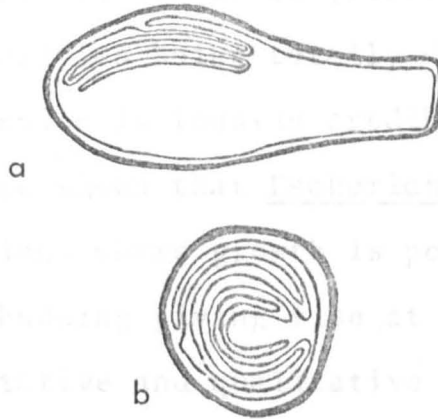


Figure 3.2

Suggested pattern of membrane development in

Rhodopseudomonas rubra. (Higgins and Shockman, 1971).

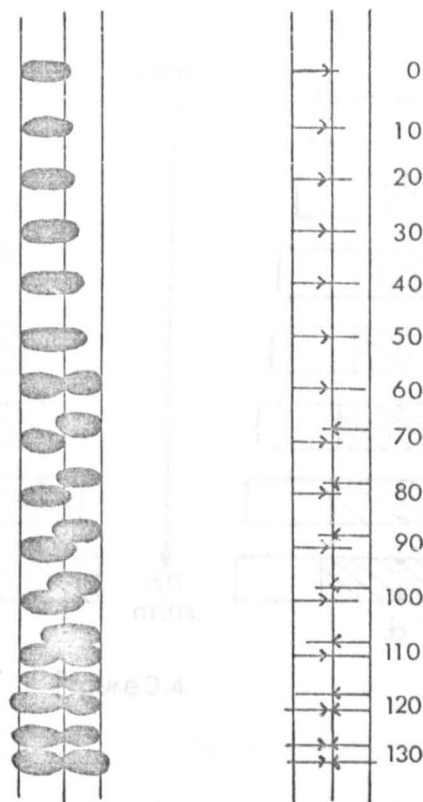
a. Postulated longitudinal section of mother cell.

b. Postulated transverse section of mother cell.

(Whittenbury and McLee, 1967).

studies on cell wall synthesis (Beachey and Cole, 1966; Rogers, 1970; Lin, Hirota and Jacob, 1971) it becomes clear that cell growth is not a symmetrical event either morphologically or physiologically and in all but a few cell types e.g. Streptococcus (Higgins and Shockman, 1971), must yield two unequal cells. In principle this is synonymous with the events occurring in morphologically extreme budding bacteria. The term budding as applied by Starr and Skerman (1965) to unequal cell division in which two siblings with obvious inequality rather than morphological equivalence are produced, although convenient, poses several serious ambiguities.

regarded simply as asymmetric growth followed by binary fission. The concept of a mode of reproduction which gives rise to two derivative cells (siblings) with obvious quantitative inequalities being totally different from 'normal' binary fission is losing credibility. Donachie and Begg (1970) have shown that Escherichia coli can be grown under conditions where growth is polar and essentially similar to budding giving rise at division to two siblings of quantitative and qualitative inequalities (figure 3.3). Further evidence of inequality, as regards division of old and new material, which in essence is argued to be the difference between budding and binary fission, has come to light from studies on the growth of the cell envelope (Rogers, 1970; Higgins and Shockman, 1971). Irrespective as to whether one considers the unit cell model of Donachie and Begg (1970) or that of Autissier, Jaffe and Kepes (1971)(figure 3.4) in conjunction with studies on cell wall synthesis (Beachey and Cole, 1966; Rogers, 1970; Lin, Hirota and Jacob, 1971) it becomes clear that cell growth is not a symmetrical event either morphologically or physiologically and in all but a few cell types e.g. Streptococcus (Higgins and Shockman, 1971), must yield two unequal cells. In principle this is synonymous with the events occurring in morphologically extreme budding bacteria. The term budding as applied by Starr and Skerman (1965) to unequal cell division in which two siblings with obvious inequality rather than morphological equivalence are produced, although convenient, poses several serious ambiguities.



Growth of the cell envelope in Escherichia coli according to two variants of the unit cell model.

Figure 3.3

a. represents cell growth according to a model in which a unit cell has a single growth zone located at one pole (Donachie and Begg, 1970). The growth of a single cell of Escherichia coli on minimal agar. The positions of the ends of the cells were measured relative to fixed markers in the agar.

b. represents the possibility that each unit cell has a central growth zone (Autissier, Jaffe and Kopes, 1972). The cell grows by elongation of one end from 0-60 mins. Division is between 60 and 70 min. Cells then slip out of alignment and each continues to grow unidirectionally from the end which was formed at the division. (Donachie and Begg, 1970).

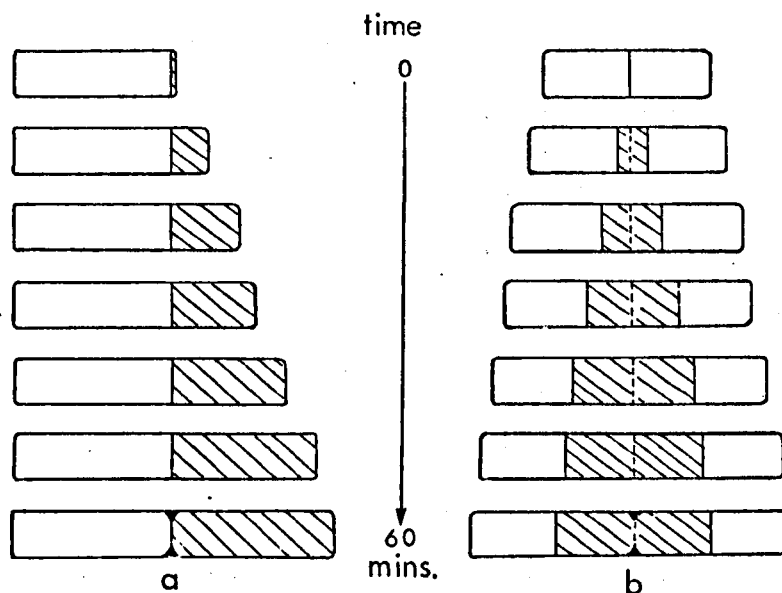


Figure 3.4

Growth of the cell envelope in Escherichia coli according to two variants of the unit cell model.

a. represents cell growth according to a model in which a unit cell has a single growth zone located at one pole (Donachie and Begg, 1970).

b. represents the possibility that each unit cell has a central growth zone (Autissier, Jaffe and Kepes, 1972).

(Donachie, Jones and Teather, 1973)

Unquestionably the most outstanding morphological characteristic of the budding bacteria is the possession of unique cellular extensions. The morphology and physiology of stalk or filament synthesis, however, has only been studied in detail by Schmidt and Stanier (1966) who concentrated on the cellular stalks of Caulobacter and Asticcacaulis species. The evidence they presented was consistent with the hypothesis that the stalk is synthesised at its junction with the cell and that the stalk wall is a relatively inert non-growing structure. There are no comparable studies of filament synthesis in Hyphomicrobium and Rhodomicrobium. Jones and Hirsch (1968) did however carry out chemical analysis of the cell walls of Hyphomicrobium showing that cell wall composition is typical of the Gram negative bacteria. Indirect evidence as to the nature of filament extension of Rhodospseudomonas palustris and Hyphomicrobium may be taken from bacteriophage absorption studies (Bosecker, Drews and Tauschel, 1972; Voelz, Gerencser, Kaplan, 1971). In both systems 'phage was found to adsorb only to the growing tip of the filament and to the developing bud. To explain these and similar findings (Boelz and Burchard, 1971) it has been hypothesised that the cytoplasm and cytoplasmic envelope is differentiated into synthetically active and less active compartments so accounting for regionally restricted 'phage adsorption and replication.

Poindexter (1964) investigated the fine structure of Caulobacter stalks showing that the core of the stalk is composed of membrane which is continuous with a unique, localized polar membrane structure. Consequently it has

been proposed that the stalk is devoid of both ribosomes and nuclear material (Schmidt and Stanier, 1966) although no evidence has been presented as to its physiological function. In contrast to this, the filaments of Hyphomicrobium and Rhodomicrobium are intimately involved in daughter cell formation. These filaments must therefore contain nuclear and cytoplasmic material destined for the daughter cell. The synthesis and movement of DNA in the budding bacteria has, however, not been investigated apart from tentative observations by Murray and Douglas (1950) on gross nuclear changes prior to and during bud formation.

Many intriguing questions concerning the budding, filamentous photosynthetics Rhodospirillum rubrum, Rh. viridis, Rh. acidophila, and Rhodomicrobium await answers. To this end isolation of strains of these microorganisms from freshwater environments was undertaken. The enrichment and assay procedures adopted yielded several strains of Rhodospirillum in addition to eight isolates of a novel Rhodomicrobium species capable of producing resting and resistant cells in adverse environments. Work was concentrated on characterising the resistant cells of Rhodomicrobium and on comparing this isolate with the original isolate of Rhodomicrobium vannielii (the type strain). Further studies were on precise characterisation of growth and replication during the vegetative cycle.

Section 3.2Materials and Methods.a) Source of organisms

Species of photosynthetic appendaged bacteria were isolated from freshwater and surface mud samples. Additional strains of Rhodospseudomonas viridis and Rhodospseudomonas acidophila were obtained from Prof. N. Pfennig (University of Gottingen) and the original isolate of Rhodomicrobium vanniellii from Prof. R. Whittenbury (University of Warwick).

b) MediaPhosphate buffer

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	15.6g
---	-------

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	35.85g
--	--------

or Na_2HPO_4	14.2g
------------------------------	-------

distilled water to 1 litre

further diluted (1:1) to give a 0.1M phosphate buffer pH 6.8.

Medium SpR

NH_4Cl	0.5g
------------------------	------

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.4g
---	------

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.05g
---	-------

NaCl	0.4g
---------------	------

Sodium hydrogen malate	1.5g
------------------------	------

Pfennig's trace element solution (Pfennig, 1969)	0.5ml
--	-------

Distilled water to 1 litre.

The pH was adjusted with KOH to 6.8 prior to autoclaving.

50 mls phosphate buffer (0.1M, pH 6.8) was added aseptically after autoclaving.

Medium PYR

This is SpR medium to which 1.5g sodium pyruvate has been added.

Complex Growth Medium.

Yeast extract	1g
SpR <u>or</u> PYR medium	1 litre.

Phage buffer.

KH_2PO_4	3.0g
Na_2HPO_4	7.0g
NaCl	4.0g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g

Distilled water to 1 litre.

The pH was adjusted to 7.2-7.6 prior to autoclaving.

Standard Saline Citrate (SSC)

NaCl	87.7g
Na citrate	44.2g

Distilled water to 1 litre.

As prepared above the SSC is 10 times concentrated and is diluted to the required concentration with distilled water.

All solid medium contained 1.5% (w/v) Bacto Difco agar.

c) Determination of Growth requirements.

Growth requirements were determined in SpR medium.

Staley's (1968) vitamin solution and an amino acid, purine, pyrimidine solution were used as broad spectrum assay solutions.

Amino acid, purine, pyrimidine solution (APP)

adenine	5mg	alanine	5mg	isoleucine	5mg
thymine	"	arginine	"	tyrosine	"
guanine	"	leucine	"	proline	"
cytosine	"	serine	"	histidine	"
uracil	"	cysteine	"	methionine	"
		tryptophan	"	glutamic acid"	
		threonine	"	lysine	"
		aspartic acid"		valine	"
per litre of distilled water.					

0.1 ml of this solution was added to 10 ml medium.

Precise auxotrophic requirements were determined by the pool method of Holliday (1956). The concentrations used were as from Clowes and Hayes (1968).

The growth factors were each made up aseptically in distilled water at the concentrations indicated. These were then combined aseptically in equal volumes to form pools and added to liquid SpR medium.

Cultures were washed thoroughly in SpR medium lacking growth factors prior to inoculation. Requirement or non-requirement was established by three consecutive inoculations into fresh medium.

d) Nitrogen source variation.

NH_4Cl , KNO_3 , Urea and Casamino Acids (0.5g/litre) were individually added to liquid SpR medium lacking nitrogen. Cultures were washed free of any fixed nitrogen and grown under an atmosphere of helium, except where gaseous nitrogen alone served as the nitrogen source.

e) Enrichment.

25 ml and 50ml screw cap bottles were inoculated with 5ml water or 0.5g mud sample, completely filled with medium and incubated at 30°C under constant illumination from tungsten lamps giving an intensity of 5-6,000 lux.

Selective enrichment by substrate variation as proposed by vanNiel (1944) was employed.

Selection of Rhodopseudomonas viridis was attempted by incubating cultures behind a deep red 'Kodak Wratten' filter allowing passage of light in the red and infra red region.

f) Isolation.

Three methods were used for obtaining pure cultures.

i) repeated application of the agar shake culture method.

ii) streak plate purification of organisms growing chemo-organically in the dark (Rhodopseudomonas palustris, Rh. acidophila and Rhodomicrobium).

iii) repeated recloning of organisms growing on agar slants prepared in 250 ml bottles which had been sealed with rubber Suba-seals and flushed with oxygen free nitrogen.

g) Maintenance of cultures.

Isolates and stock cultures were maintained in stab cultures of PYR medium in 1/4 oz. vials, incubated at 30°C for five days and stored in the light at room temperature thereafter.

Cultures were transferred once every four months.

h) Experimental growth conditions.

Cultures were grown in a variety of glass vessels which were sealed with rubber Suba-seals, flushed with oxygen free nitrogen and incubated at 30°C under constant

illumination from tungsten lamps (8,000 lux.). Heat generated by the lamps was dispersed by a fan.

The required atmosphere in a culture vessel was obtained by passing oxygen free nitrogen through a 0.22 μ Millipore filter and via a sterile syringe needle, inserted in the Suba-seal, into the flask. A second syringe needle served as a gas outlet. Flushing was continued until the oxygen content approached zero, as determined chromatographically.

i) Chromatographic evaluation of gaseous environments.

Hollis (1966) reported that nitrogen, oxygen, nitric oxide, carbon dioxide, nitrous oxide and several other gases are resolved at room temperature by gas chromatography on polyacromatic beads. More recently Herbert and Holding (1972) and Payne (1973) have published data on the separation and estimation of gases produced or utilized by microorganisms using in the former Porapak Q and in the latter silica gel, molecular sieve or Porapak R as the column packing material. When either of these methods was employed difficulty was encountered in that the apparatus, although similar to that used by Herbert and Holding (1972) would not maintain a steady 30°C which resulted in serious drifting of the recorder base line but more importantly in neither method was there sufficient separation of the oxygen and nitrogen peaks so prohibiting accurate quantification of the oxygen concentration (figure 3.5).

To surmount these problems the following chromatographic procedure was formulated:

A Pye GC104 series (Model 34) chromatograph fitted with a thermal conductivity detector and connected to a

Figure 3.5

Separation of a known mixture of gases by chromatography using Porapak Q with helium at a flow rate of 45 ml/min. and 28°C, a filament current of 150 mA and a recorder chart speed of 2.54 cm/min.
(Payne, 1973).

Figure 3.6

Chromatographic separation of Air, CH₄ and CO₂ using Porapak R with helium at a flow rate of 30 ml/min and 50°C, a bridge current of 240 mA and a recorder chart speed of 10 mm/min.

Figure 3.7

Chromatographic separation of O₂, N₂ and CH₄ using Molecular sieve and with other chromatographic parameters as per figure 3.6 . The recorder chart speed was 5 mm/min.

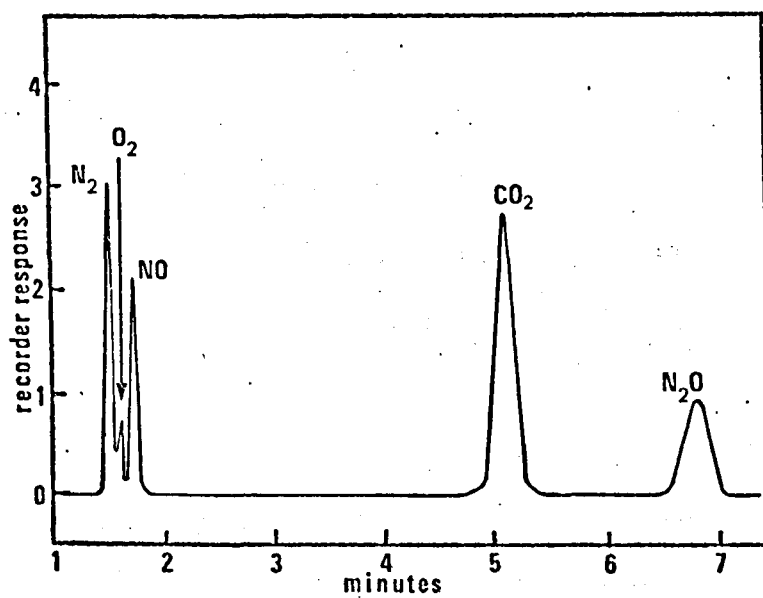


Figure 3.5

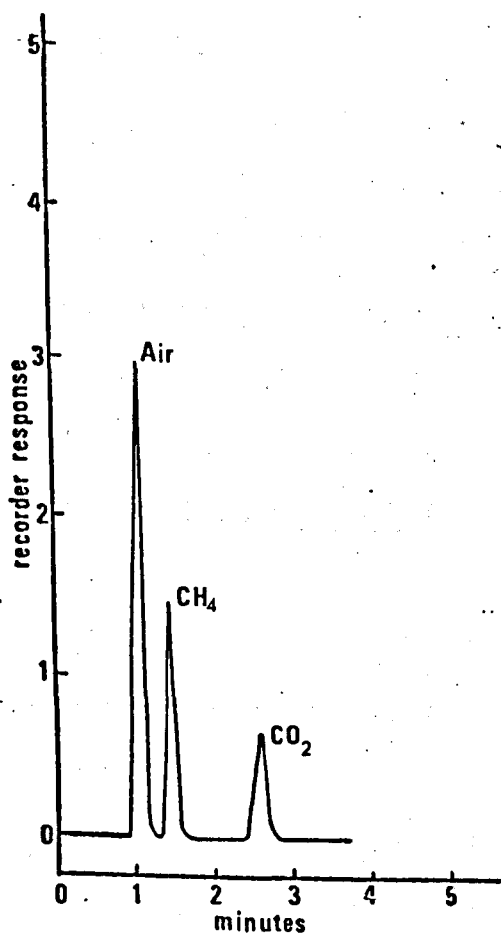


Figure 3.6

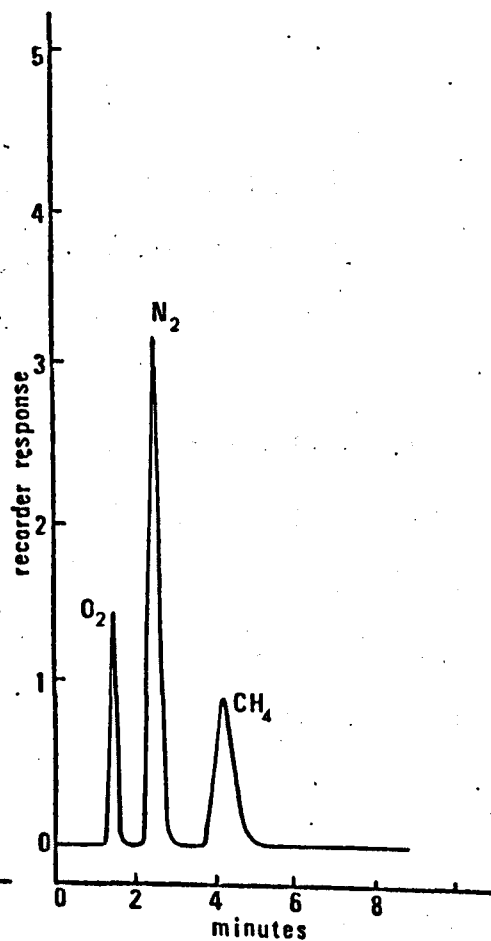


Figure 3.7

Servoscribe 1mV single span pen chart recorder was used routinely. High purity Helium (Air products) was used as a carrier gas at an inlet pressure of 251b/in² and an outlet flow rate of 30 ml/min. Glass columns (2.1m x 6mm) packed with 80-100 mesh molecular sieve 5A (Phase Separation Ltd.) and a second with 80-100 mesh Porapak R, both of which were conditioned by purging at 150°C for 3 hr. with a carrier flow rate of 50 mls/min., were used as the stationary chromatographic phases. The system was operated at a temperature of 50°C with a detector bridge current of 240mA. Typical gas separations are shown in figures 3.6 and 3.7. The minimum detection limits of separated gases were of the same magnitude as those detailed by Herbert and Holding (1972).

j) Methods of measuring growth.

Growth was followed spectrophotometrically in a Pye Unicam series II Sp500 spectrophotometer at 540 nm (protein) and at the absorption maximum of the species specific chlorophylls.

Cellular protein was determined quantitatively by the procedure of Lowry et al (1951). 5 ml samples of the cell suspension were centrifuged at 6,000 g for 15 minutes, resuspended in 1 ml of N NaOH at 37°C for 1 hr. and 0.5 ml used for the protein estimation using bovine serum albumin as the standard.

k) Absorption spectra of intact cells.

The in vivo absorption spectra of whole cells resuspended in saturated sucrose was determined spectrophotometrically using a Pye Sp500 spectrophotometer or a Pye Sp800 split beam spectrophotometer.

1) Extraction and analysis of chlorophylls and carotenoids.

Cells in the exponential growth phase were harvested by centrifugation at 6,000 g for 20 minutes and the sediment extracted and saponified either by the method of Schmidt (1971a) or that by Jensen et al (1964).

Following extraction the pigments were dissolved in petroleum ether and separated on precoated silica gel (Kieselgel F 254) thin layer plates using one of the following solvent systems depending on the polarity required.

Ethyl Acetate:Hexane (1:3 v/v)

Acetone:Petroleum Ether (1:9 v/v)

Hexane:Diethyl Ether:Glacial Acetic (35:15:5 v/v)

Ethyl Acetate:Petroleum Ether (1:10 v/v)

Individual pigment bands were removed from the chromatographic plates and resuspended in acetone prior to characterisation.

Visible and ultraviolet absorption spectra were recorded using a Pye Sp800 split beam spectrophotometer fitted with a scale expander serving a 10 mV Servoscribe recorder.

m) Extraction and analysis of cytochromes.

The cytochrome extraction and analysis was according to the method proposed by Rieske (1967).

n) Nitrogen fixation.

Fixation of atmospheric nitrogen was determined by the acetylene reduction assay (Postgate, 1972).

o) Guanine plus cytosine content of DNA determined by temperature profile analysis (T_m).

The T_m of high molecular weight DNA was determined by the procedure described by Mandel and Marmur (1968).

p) Spheroplast formation.

Lysozyme and penicillin spheroplasts of Rhodomicrobium and Rhodopseudomonas palustris were produced by modification of the technique described by Schmidt and Stanier (1966). Cells were suspended in medium consisting of PYR medium lacking added $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ but supplemented with 0.05M Tris pH 7.8, 7% (w/v) polyethylene glycol and containing 10 ug/ml freshly prepared lysozyme. Incubation was at 30°C under constant illumination (7,000 lux). The reaction was stopped at intervals by the addition of 1% (w/v) MgCl_2 and incubation continued for up to 2 hrs. Samples were examined by phase contrast light microscopy and electron microscopically by metal shadowing. Penicillin spheroplasts were formed in PYR medium supplemented with 7% (w/v) polyethylene glycol and containing 4,000 units/ml Penicillin G. The experimental procedure was similar to that used for lysozyme spheroplast formation with the exception that the reaction was stopped by the addition of penicillinase.

q) Autoradiography.

Cells were grown under the desired experimental conditions in the presence of or pulsed with L-(4-5-³H)Leucine. 0.25-0.5ml samples were taken at intervals, washed three times in phosphate buffer containing 50 µg/ml of carrier, a smear made on a clean slide, air dried and fixed by passing twice through a bunsen flame. Slides were coated with autoradiographic emulsion (Kodak NTB2) according to the method of Rogers (1969). Exposure was for a period of 2-3 weeks at 4°C in a light tight plastic box containing a few grains of silica gel. Autoradiographs were developed in a 1:1 solution of Kodak D-19 developer and distilled water and fixed for 5 minutes in Kodafix.

The developed autoradiographs were prepared as wet mounts with the coverslip sealed to the slide using a paraffin wax, vaseline (1:1 w/w) sealant. Examination was by phase contrast microscopy.

r) Separation of resting cells from heterogeneous cultures.

Separation of the resting, angular cells from heterogeneous populations was carried out by centrifugation (MSE 6 x 250 rotor, 15,000 g for 15 min.), the vegetative cells sedimenting first, the spores forming a loosely packed second layer, with subsequent purification by filtration through a glass wool column (section 4.2). Such preparations were essentially free of ovoid filamentous vegetative cells.

s) Sucrose gradients.

Linear sucrose gradients were made by the method of Britten and Roberts (1960).

t) Dipicolinic acid assay.

The method of Jansen, Lund and Anderson (1958) was used.

u) Resistance of Rhodomicrobium spores.

A sporulating culture of Rhodomicrobium was harvested, purified as above (r) and washed thoroughly in 0.01M phosphate buffer (pH 6.8).

Heat resistance

Cultures prepared as described above were heat treated through a range of temperatures (50-100°C) in 2 ml thin walled glass tubes (freeze drying ampoules) which were totally immersed. 0.1 ml samples were taken at intervals, serially diluted and 0.1 ml from each dilution was inoculated into liquid and agar shake tubes of SpR medium with subsequent incubation at 30 C under constant illumination.

Ultraviolet light.

5 ml of the prepared spore sample, in a glass petri dish, was irradiated with light of a wavelength of 257 nm. Samples were removed at intervals and viable numbers determined as described above. Cell aggregation and shielding was prevented by continuous agitation of the plate. An exponential culture of E. coli (C603), treated similarly to the spore culture, was used as a standard.

Lysozyme resistance.

A spore suspension was incubated at 30°C in the presence of 4 mg/ml freshly prepared lysozyme in Tris-buffer (section 5.2). This was compared microscopically with a vegetative culture treated in a similar manner.

v) Phage isolation.

Samples of filtered sewage, surface mud sediment and freshwater were diluted with a complete growth medium (PYR) and heavily inoculated with Rhodomicrobium to a final volume of 2 litres. Incubation was at 30°C under constant illumination on a rotary shaker.

After incubation the cultures were centrifuged at 6,000 g and the supernatant filtered through Millipore filters (0.22 µ). Aliquots of the cell free filtrate were centrifuged at 100,000g and the sediment resuspended in phage buffer. Serial 10 fold dilutions were made and tested for phage activity. 0.1 ml of each dilution was inoculated into 2 mls of swarm cell culture and incubated for several hours so avoiding stages of immunity to phage infection during the life cycle of the host. The culture was then mixed with 10 mls PYR agar medium, plated out and overlayed with a thick layer of agar medium (to generate a microaerophilic environment). Incubation was at 30°C under constant illumination.

A few plaques ranging from 1-3mm in diameter were observed after 3-4 days. These were picked and propagated.

w) Isolation and purification of flagella.

A 10 litre culture of fermenter grown Rhodomicrobium cells which had reached the late lag phase was centrifuged at 6,000 g for 20 minutes. The cells were discarded and an 80% (w/v) ammonium sulphate precipitation carried out on the supernatant culture fluid. This was filtered through a 0.22 μ , 45mm Millipore membrane filters, the precipitate washed off into 0.01M Tris (hydroxy-methyl) aminomethane (Tris) buffer pH 7.1 and washed three times by high speed centrifugation to remove the ammonium sulphate. The flagellar pellet was finally taken up in 0.01M Tris pH 7.1 and purified by equilibrium sedimentation in CsCl as described by Shapiro and Maizel (1973).

x) Gel electrophoresis.

A 0.5mg amount of protein was made 10% with respect to trichloroacetic acid (TCA), the resulting precipitate was collected, washed twice with 5% (w/v) TCA and once with acetone. The sediment was dissolved in 0.2ml of a solution containing 1% (w/v) sodium dodecyl sulphate (SDS), 0.1% (v/v) 2-mercaptoethanol in 0.05M Tris buffer pH 7.8. The solution was heated at 100°C for one minute to disrupt possible metastable aggregates (Shapiro and Maizer, 1973).

To 1ml of the sample was added 0.1ml of 0.1% (w/v) bromophenol blue plus 10% (w/v) sucrose.

Section 3.3 Results and Discussion.

A. Enrichment and isolation.

Since the early works on the photosynthetic bacteria by van Niel (1944) and Giesberger (1947) the literature has been supplemented with comprehensive reviews on the 'biology' of photosynthetic bacteria by Pfennig (1967) and on their physiology by Stanier (1961) and Lascelles (1968).

It was van Niel (1931,1944), however, who worked out the rational enrichment procedures for photosynthetics, in particular for the photosynthetic purple sulphur (Thiorhodaceae) and non-sulphur purple bacteria (Athiorhodaceae) indicating the selective pressures ensuing from variation in cultural parameters, e.g. carbon source variation, sulphide concentration, pH and light intensity. The photosynthetic purple sulphur bacteria are enriched by taking advantage of their ability to grow photolithotrophically utilising sulphide, inhibitory in high concentrations to Athiorhodaceae, as the electron donor. Consequently in Athiorhodaceae enrichments, sulphates are almost completely replaced by the corresponding chlorides so eliminating the development of sulphate reducing bacteria and in turn sulphur photosynthetics.

The appendaged, budding obligatory life-cycled Athiorhodaceae have been enriched and isolated by various workers: Rh. palustris by van Niel (1931); Scher, et al (1963), Whittenbury and McLee, (1967); Rh. viridis by Eimhjellen et al (1963), Drews and Giesbrecht (1966); Rh. acidophila by Pfennig (1969); and Rm. vanniellii by Duchow and Douglas (1949), Hirsch and Conti (1965). All these organisms have been enriched on media differing in complexity and containing a wide range of carbon sources.

Modification and variation of the carbon source, pH value, light intensity and wave length were applied to enrichments in an attempt to selectively enrich the desired non-sulphur purple photosynthetics. A simple mineral base, a modification of that proposed by Pfennig (1969), containing NH_4Cl as the nitrogen source, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaCl , inorganic phosphate, trace elements and a vitamin solution (Staley, 1968) or yeast extract was supplemented with various carbon sources over a range of pH values. Under photosynthetic cultural conditions and using the variable parameters described above, species of Athiorhodaceae were enriched and subsequently purified by the standard techniques (Table 3.1).

Enrichment and isolation of Rhodospseudomonas palustris is possible on a wide variety of carbon sources over a pH range of 6.5-7.5. It is the most consistent isolate from Athiorhodaceae enrichments, an observation explained by its diverse physiological capabilities, i.e., it is capable of photolithotrophic, photoorganotrophic and chemoorganotrophic growth and, additionally, will tolerate low oxygen tensions when growing photosynthetically. In contrast, its green counterpart, Rh. viridis, is an obligate photoorganotroph (Drews and Giesbrecht, 1966) and consequently proved more difficult to isolate than Rh. palustris. Eimhjellen (1967) successfully used infra-red filters to selectively enrich Rh. viridis, the in vivo wavelength absorption maxima of the bacteriochlorophyll-b being 1020-1030nm. This technique was applied without success. However, one strain was obtained by straightforward enrichment and isolation.

Rhodospseudomonas acidophila, characterised by Pfennig

Table 3.1

The effect of carbon source and pH variation on the enriched species of photosynthetic bacteria.

Added carbon source	optimum pH	incubation time (weeks)	primary enriched species.
methanol	6.8	3-4	<u>Rh. palustris</u>
ethanol	6.8	4	undetermined
benzoate	6.8	3	<u>Rh. palustris</u> <u>Rhodospirillum</u>
p aminobenzoate	6.8	3	<u>Rhodospirillum</u>
malate	6.8	1-2	<u>Rh. palustris</u> <u>Rh. viridis</u> <u>Rhodomicrobium</u>
"	5.5	1-2	<u>Rh. acidophila</u> <u>Rhodomicrobium</u>
formate	6.8	3-4	undetermined
acetate	6.8	3	<u>Rh. palustris</u>
pyruvate	6.8	2-3	<u>Rh. palustris</u> <u>Rhodospirillum</u>
"	5.5	2-3	<u>Rh. acidophila</u>

(1969) grows optimally at pH ranges between 5 and 6. Consequently, this species was only enriched in cultures with pH values below 6. Malate and pyruvate at low pH gave consistent preferential enrichment of photosynthetic bacteria closely resembling Pfennig's isolate.

The consistency with which Rhodomicrobium species were enriched using sodium hydrogen malate is of considerable importance when consideration is given to the literature (Duchow and Douglas, 1949; Murray and Douglas, 1950; Conti and Hirsch, 1965; Hirsch and Rhienheimer, 1968). There is no record of specific enrichment cultural conditions. The listed procedure (Table 3.1) gives a greater than 50% success rate affording ease of isolation and purification by the routine procedures.

The selective factor(s) of the malate enrichments cannot be determined, due to the complexity of the inoculum, but it may be pertinent to suggest that the assimilation of malate, which involves the endogenous production of CO_2 and pyruvate (Kondrat'eva, 1965) provides not only reducing power but a readily available CO_2 source removing the necessity for exogenous CO_2 uptake. All media used to date for the growth of Athiorhodaceae isolates have required the addition of bicarbonate or an enriched atmosphere of CO_2 (Trentini and Starr, 1967). This is not required with malate as the carbon source.

B. Occurrence and distribution.

All anaerobic aquatic environments tested yielded Athiorhodaceae isolates on enrichment. Their presence has also been shown in salt water and moist terrestrial soils (Hirsch and Conti, 1965; Hirsch and Rheinheimer, 1968; Herbert, pers. comm.).

C. Rhodomicrobium (Rm5) vs. Rhodomicrobium vannielii (type strain)

(i) Morphology

Unlike Rh. palustris, Rh. viridis (Whittenbury and McLee, 1967) and Rh. acidophila (Pfennig, 1969), the morphological and developmental characteristics of Rhodomicrobium species reported in the literature present a complex picture with many ambiguities and little substantiated information. (Duchow and Douglas, 1949; Murray and Douglas, 1950; Douglas and Wolfe, 1959; Conti and Hirsch, 1965; Gorlenko, 1969). When originally isolated Rm. vannielii was reported as being non-motile (Duchow and Douglas, 1949). Douglas and Wolfe, (1959) however, substituting sodium lactate for ethanol as the carbon source, not only increased the growth rate (no figures given) but found that coccoid peritrichously flagellated cells appeared in young cultures. The developmental pattern has therefore been described in part i.e. bud formation from the stalked mother cell which upon maturity remain attached, hence chain formation, but nothing is known as to the construction of these sequences or the developmental pattern of the motile cells.

A Rhodomicrobium isolate described by Gorlenko (1969) grows vegetatively by chain formation but in addition produces angular resting cells in old cultures. Such structures were undoubtedly originally observed in the initial isolate by Murray and Douglas (figure , 1950) but received no further comment being explained as sessile non viable entities most probably because they appeared infrequently and in very low numbers. Gorlenko (1969) reported no motility in his isolate. The investigation was, however, superficial and failed to consider the overall growth cycle, therefore the existence of motile swimmers in this strain cannot be ruled out.

The Rhodomicrobium isolates obtained during the course of this study formed all three cell types, stalked mother cells with bud and concomitant chain formation, motile swarm cells and angular resting cells, all in strict accord with the growth curve (figure 3.8).

The question therefore arises, have these observations been made on one genus with species variation or simply on one species subjected to different physiological conditions which are expressed by cell morphological and functional variance?

Rhodomicrobium (Rm5), the isolate used primarily throughout this study, and Rm. vanniellii are closely related with regard to cultural characteristics. Grown photosynthetically on PYR medium (section 3.2) the pigmentation of both is of a deep orange red colouration. Phase contrast microscopic (plate 3.1) and electron microscopic examination of cultures in the exponential growth phase reveals the morphological, developmental and dimensional similarities of both. Motile cells are consistently present in both cultures during the growth phase and the strains are morphologically indistinguishable (plate 3.2). They bear little resemblance, however, to the motile cells reported by Douglas and Wolfe (1959) but are descriptively similar to the ovoid cells of a marine Rhodomicrobium strain isolated by Trentini and Starr (1967) but which was unfortunately not further characterised.

Although comparable during lag and exponential growth, Rhodomicrobium (Rm5) and Rm. vanniellii differ markedly at the onset of the stationary phase. Rhodomicrobium (Rm5) initiates angular cell formation in profusion (plate 3.3) while in Rm. vanniellii cultures the filaments become highly

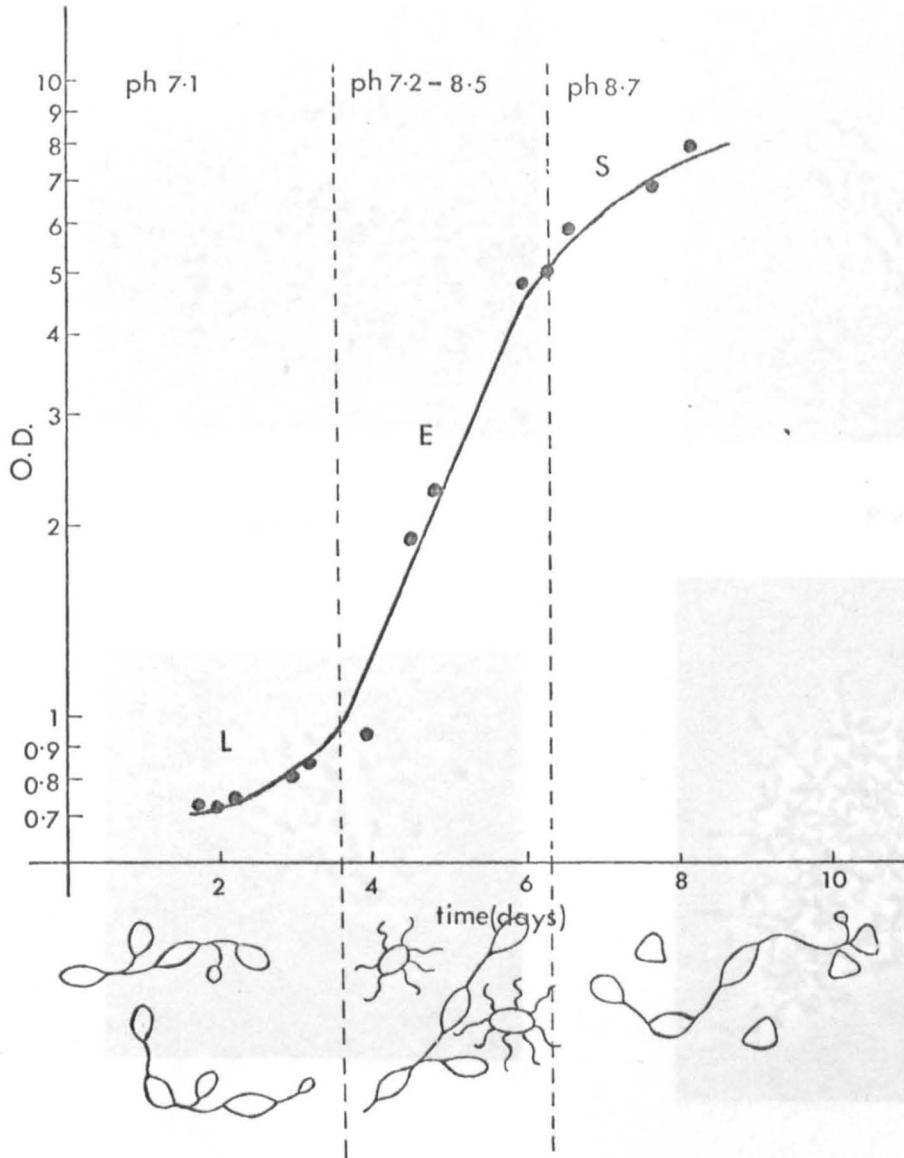
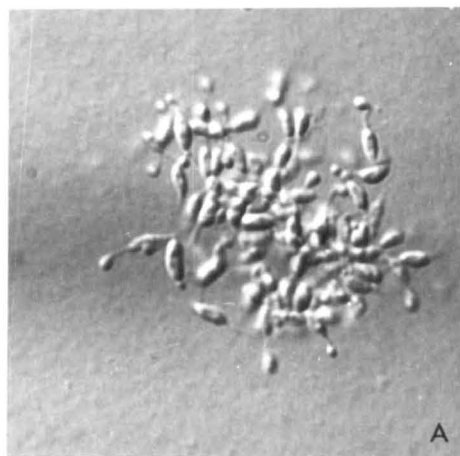


Figure 3.8

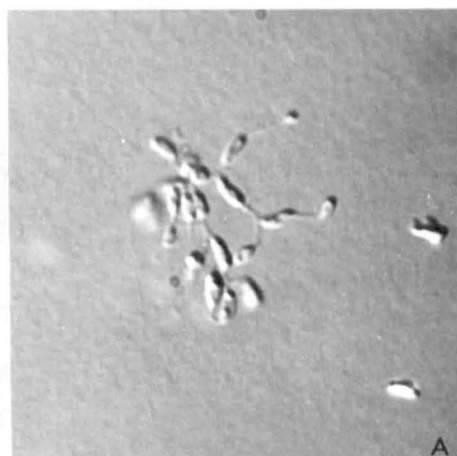
Growth curve of *Rhodomicrobium* (Rm5) grown in a batch fermenter (2,000 lux, PYR medium) showing the morphological cell types and pH variations over a period of days.

(L, lag phase; E, exponential phase; S, stationary phase)

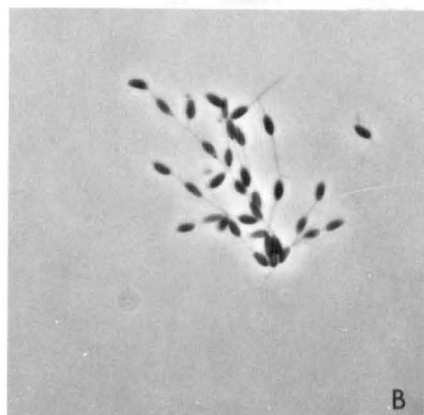
showing developmental, morphological and dimensional similarities. (x 1100)



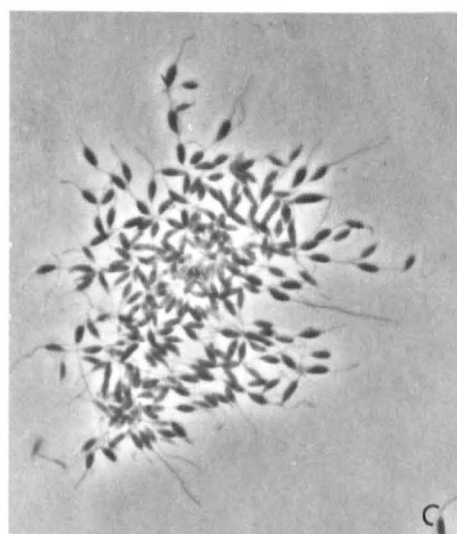
RM5



RV



B



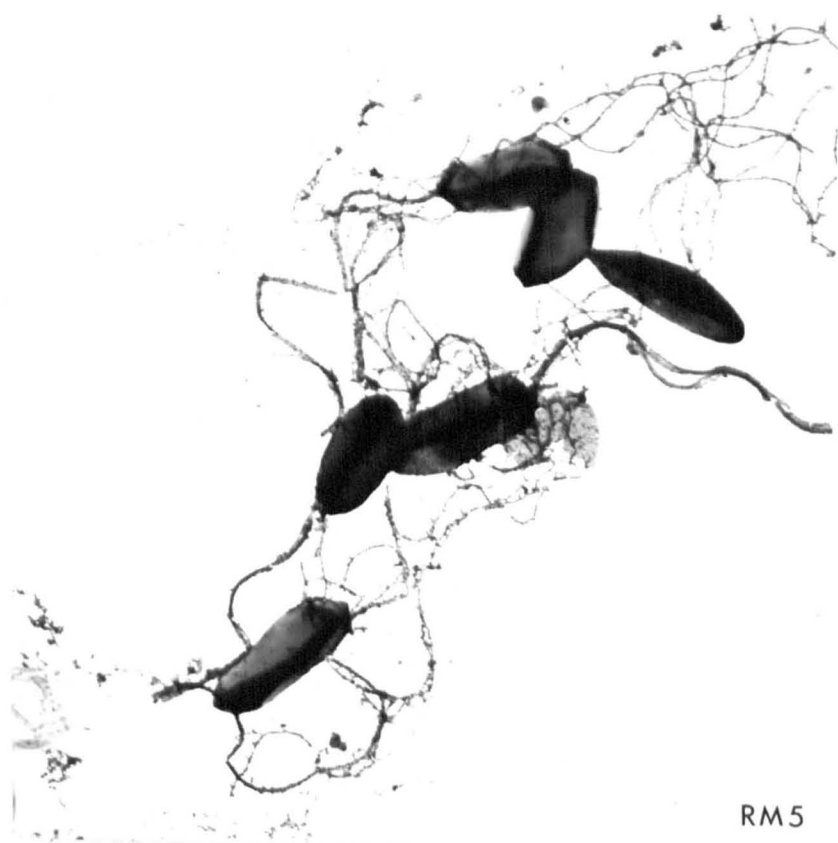
C

Plate 3.1

Interference (A) and phase contrast (B,C) photomicrographs of Rhodomicrobium (Rm5) and Rhodomicrobium vanniellii (RV) showing developmental, morphological and dimensional similarities. (x 1100)

Plate 3.2

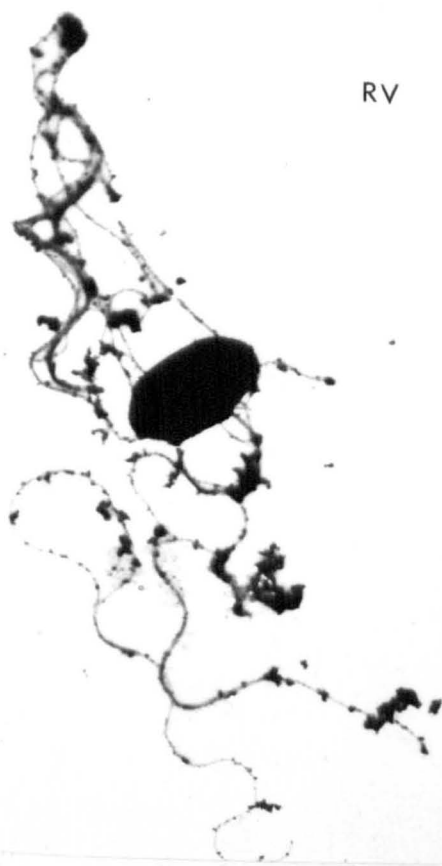
Negatively stained (uranyl acetate) electron micrographs of Rhodomicrobium vanniellii showing peritrichous flagellation and overall morphology. (x 12000)



RM5

cells in a
(Rm5) (x1100)

RV

Plate 3.2

Negatively stained (uranyl-acetate) electron micrograph of Rhodomicrobium(Rm5) and Rhodomicrobium vannielii swarm cells showing peritrichous flagellation and ovoid morphology. (x 12000)

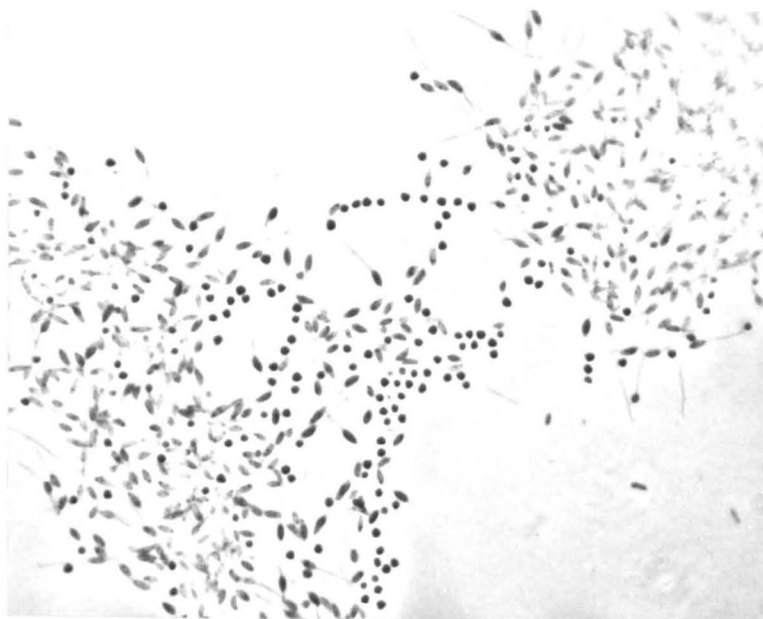


Plate 3.3

Phase contrast photomicrograph of angular cells in a stationary phase culture of Rhodomicrobium(Rm5) (x1100)

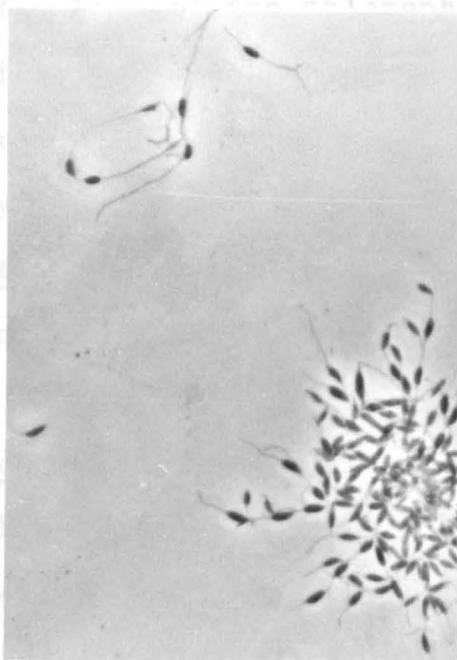


Plate 3.4

Multiple, sessile branch formation in a stationary phase culture of Rhodomicrobium vannielii. Phase contrast (x 1100).

branched, cells translucent and susceptible to lysis and only very infrequently are cells encountered which resemble the angular cells of Rhodomicrobium (Rm5) (plate 3.4).

(ii) Photosynthetic pigment analysis.

Pigment analyses were made on Rhodomicrobium (Rm5) and Rm. vannielii cells grown on pyruvate malate medium at an initial pH of 7.0 and a light intensity of 800-1,000 lux.

The absorption spectra of intact cells of both organisms are identical showing the maxima of bacteriochlorophyll-a containing microorganisms, (596, 807, 875 and a shoulder between 890 and 900nm) (Jensen et al, 1964; Schmidt et al, 1965), together with carotenoid maxima at 460, 492, and 526 nm. Typical in vivo spectra are given in figure 3.9.

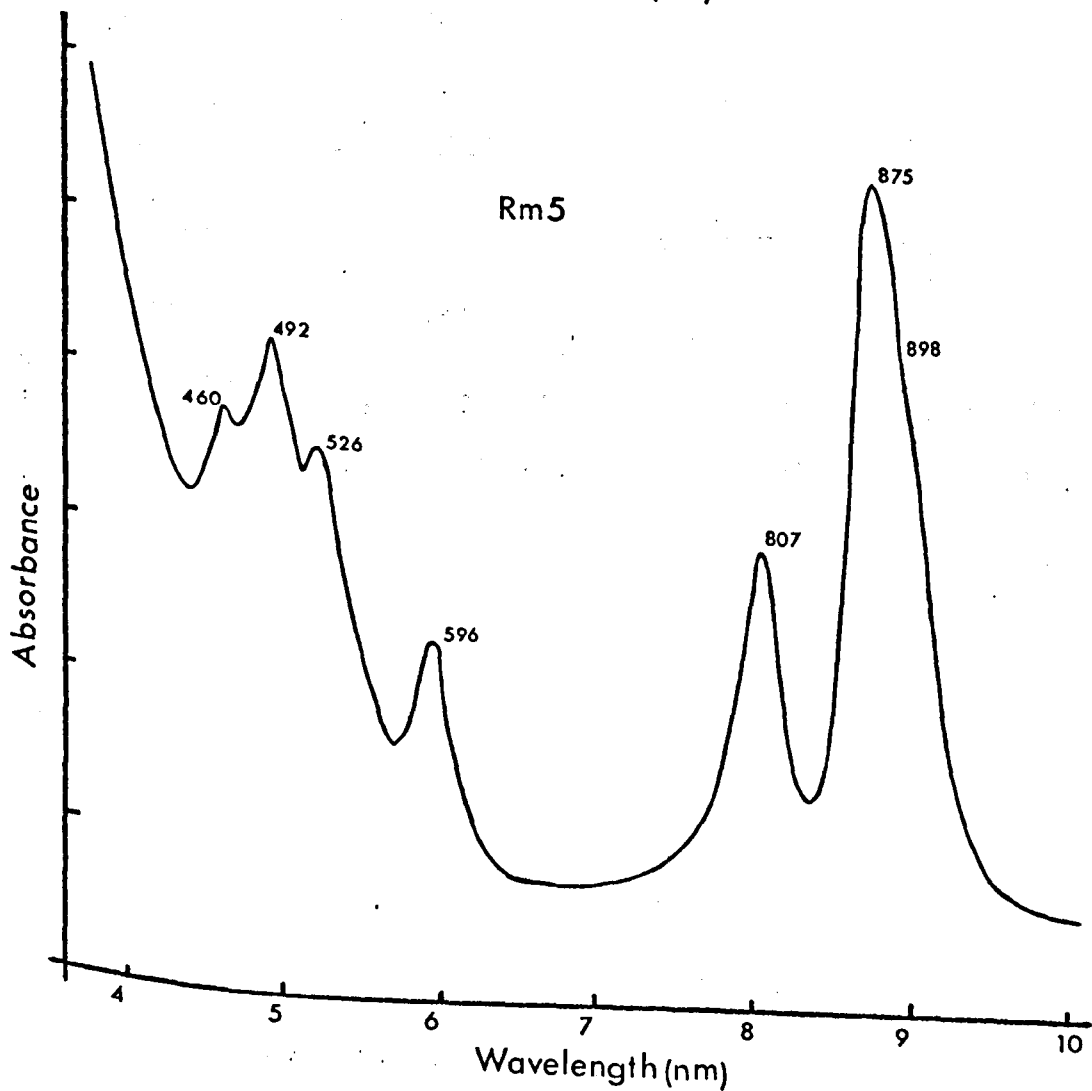
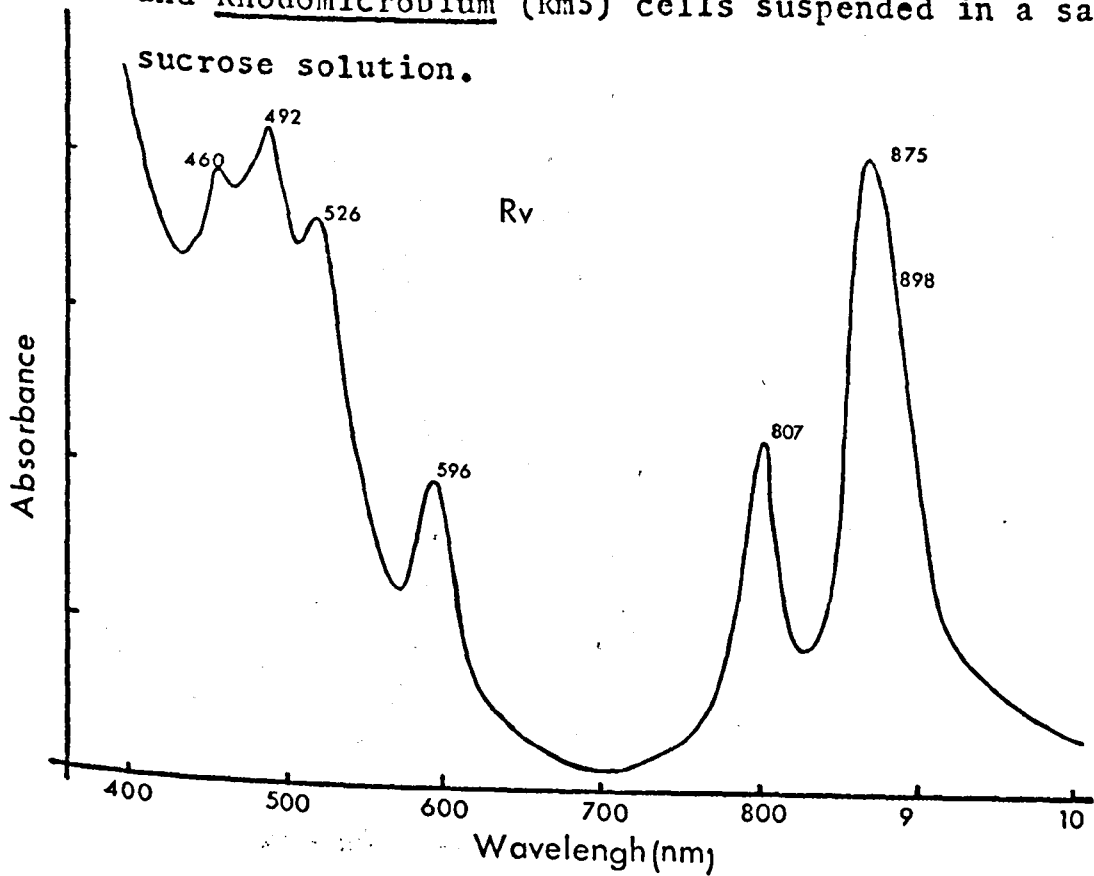
a) Chlorophyll.

The identification of the chlorophyll of Rm. vannielii by Katz and Wassink (1939) was based on a comparison of its absorption spectrum in alcohol with a crude spectrum of bacteriochlorophyll-a. No absorption or other details were given. Jensen et al (1964), however, carried out extensive analyses of the chlorophylls of photosynthetic bacteria and encountered only bacteriochlorophyll-a in Rm. vannielii and the Athiorhodaceae in general, with the exception of Rh. viridis which contains bacteriochlorophyll-b.

In vitro (ether solution) bacteriochlorophyll-a has a red absorption band at 773 nm, and an orange absorption band at 575nm. In vivo the red band is shifted markedly to the near infra-red. This is illustrated by the absorption spectra of a suspension of whole cells of Rhodomicrobium (Rm5) and of the bacteriochlorophyll extracted by ether from these cells (figure 3.10).

Figure 3.9

In vivo absorption spectra of Rhodomicrobium vannielii and Rhodomicrobium (Rm5) cells suspended in a saturated sucrose solution.



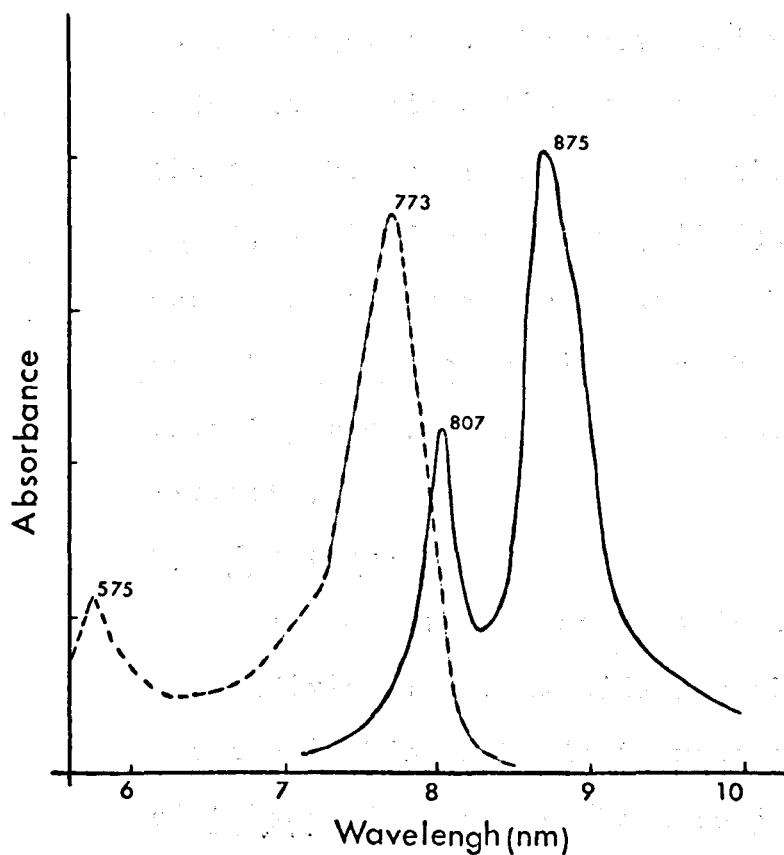


Figure 3.10

Absorption spectra of the bacteriochlorophyll of Rhodospirillum (Rm5).

Solid curve, saturated sucrose suspension of whole cells.

Broken curve, ether extract of the same cells.

Although it is important to realise that there is no longer a simple correlation between the recognised major taxonomic entities among photosynthetic bacteria and the type of chlorophyll which they contain (Eimhjellen et al, 1963; Jensen et al, 1964). Bacteriochlorophyll-a was encountered exclusively in Rhodomicrobium (Rm5) not only by in vitro and in vivo absorption analysis but by thin layer chromatography of concentrated acetone:methanol extracts.

b) Carotenoids.

The carotenoid composition, the chemical structure of the individual carotenoids and the pathways involved in carotenoid biosynthesis in most of the described species of Athiorhodaceae have been subjected to extensive study (Liaaen Jensen, et al, 1961; Eimhjellen and Liaaen Jensen, 1964; Liaaen Jensen, 1965; Schmidt, 1971; Liaaen Jensen and Andrewes, 1972). On the basis of the chemical structures and the biosynthetic pathways of their carotenoids the Athiorhodaceae can be divided into two categories, namely those producing carotenoids belonging to:

- a) the normal spirilloxanthin series
- b) The alternative spirilloxanthin series and the corresponding keto-carotenoids of the spheroidenone type.

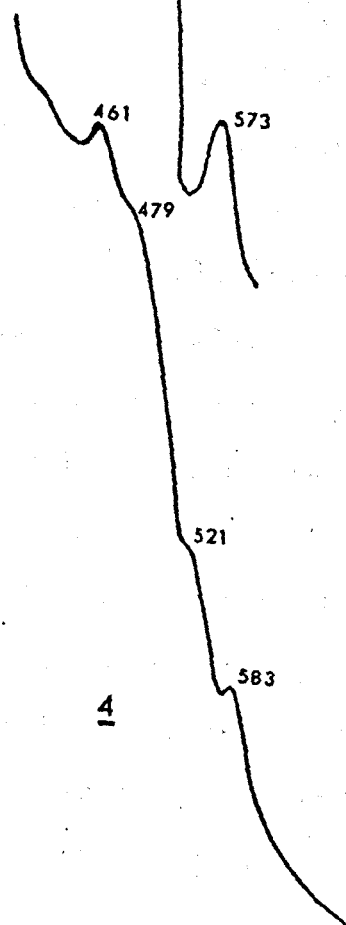
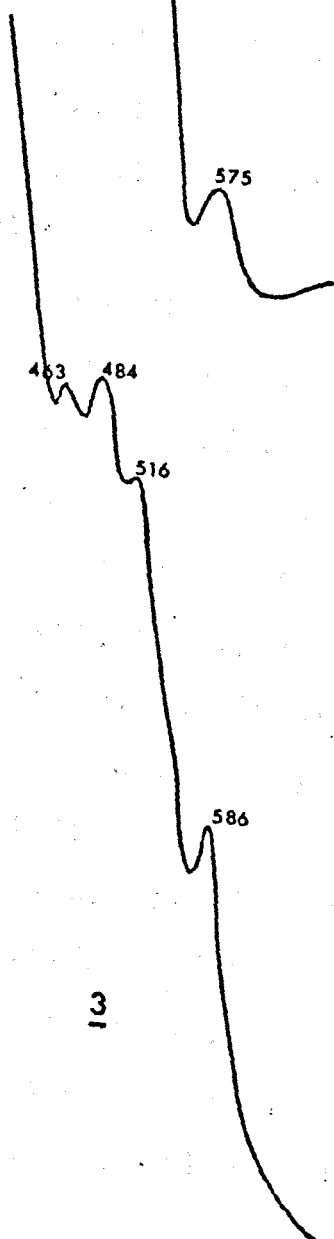
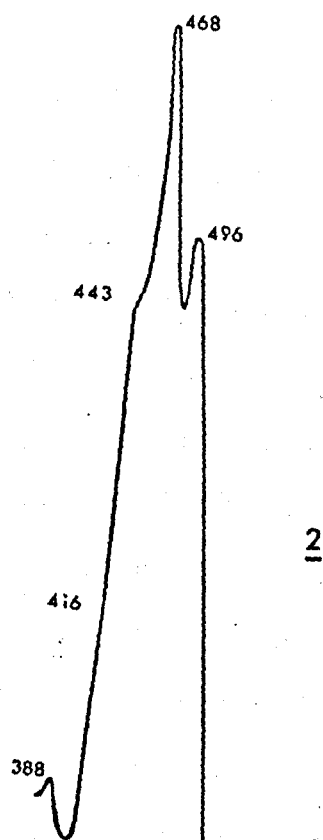
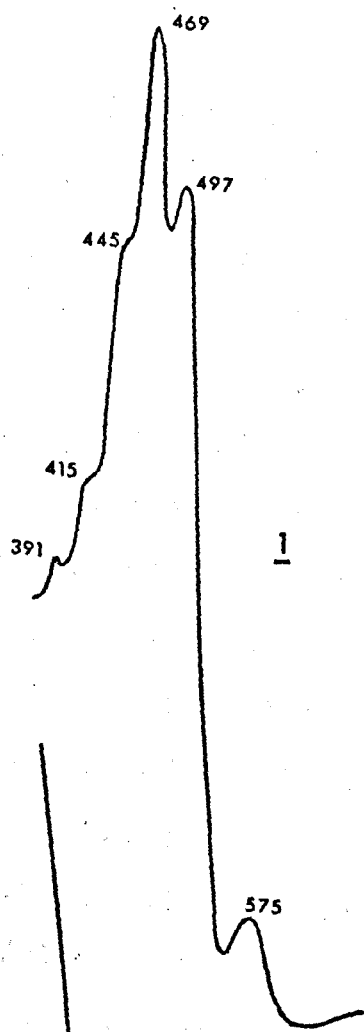
The initial works on the carotenoids of Rm. vanniellii (Volk and Pennington, 1950; Conti and Benedict, 1962) were of a tentative qualitative nature involving no direct comparison with authentic carotenoids, with the exception of β -carotene. Ryvarden and Liaaen Jensen (1964), however, produced not only a qualitative but a detailed quantitative study of the carotenoid composition.

Figure 3.11

Absorption spectra of the carotenoids of Rm. vanniellii(1 and 3)
and Rhodomicrobium(Rm5)(2 and 4).

Saturated sucrose solution (3 and 4).

Carotenoids suspended in ether (1 and 2).



The carotenoids of Rm. vanniellii and Rhodomicrobium (Rm5) were extracted from cultures growing exponentially on SpR medium, using a 2:7 (v/v) methanol:acetone mixture and the extract assayed qualitatively. Figure 3.11 gives the comparison of the extracted carotenoids of Rm.vanniellii and Rm5 in addition to the in vivo spectra of whole cells saturated in sucrose. The comparison of the absorption spectra of the extracts and the whole cell spectra show a close qualitative similarity between the two organisms.

Further characterisation of the carotenoid extracts was carried out by thin layer plate chromatography (TLC) using Silica gel as the stationary phase and a range of solvents differing in their eluotropic potential. A 10:1 (v/v) petroleum ether:ethyl acetate mixture was found to give the best separation and resolution. Table 3.2 summarises the data obtained using this system. (The absorption spectra of the individual bands were determined by elution from preparative TLC plates into acetone and analysis in a split beam recording spectrophotometer). Correlation of colour, absorption maxima and R_f values allowed identification of the individual carotenoids with a reasonable degree of certainty, only β carotene however being examined with respect to 'authentic' carotenoids.

These findings can be correlated closely with those of Ryvarden and Liaaen Jensen (1964). The data indicates that both organisms are identical with respect to their photopigments, i.e. chlorophyll and carotenoids, the carotenoids belonging to the normal spirilloxanthin series.

Table 3.2

Colouration, absorption maxima (acetone) position relative to R_F values and probable identification of individual carotenoids of Rhodomicrobium (Rm5) and Rhodomicrobium vanniellii.

Bands in order of decreasing R_F value.	Colour of band.	Abs.max. in acetone (nm)			Identification
a	yellow	448	476		Carotene
b	orange	444	472	502	lycopene
c	yellow		-		-
d	red-purple	468	496	525	spirilloxanthin
e	yellow-orange	446	472	502	rhodopin
f	red-orange	460	485	420	rhodovibrin
g	orange	445	470	500	1,2,1',2'tetra- hydro-1,1'-di- OH-lycopene.

(iii) Characterization of the DNA.

The DNA extracted from exponentially growing, photo-synthetic Rm. vanniellii and Rhodomicrobium (Rm5) cells was subjected to (a) neutral Caesium chloride (CsCl) buoyant density centrifugation and (b) temperature profile analysis.

(a) Individual analytical ultracentrifugation determinations of the guanine plus cytosine (G+C) mole percentages gave figures of 62.5 and 63% respectively (section 5.3). Analysis of both DNAs on the same CsCl gradient gave only one band (figure 3.12) confirming the close similarity that exists between the G + C mole percentages of the two DNAs.

(b) The temperature profile analyses gave characteristic thermal denaturation curves, indicative that there is one DNA population i.e. the G+C content is distributed about the mean value in a normal fashion which is not so with mixtures of DNA differing in G+C content (figure 3.13). The value obtained for the G + C mole percentages, 62.5% being similar to those obtained by CsCl neutral density gradient determinations precludes the possibility of unusual bases being present.

(iv) Physiology.

(a) Vitamin requirements.

The vitamin requirements of the Athicrhodaceae vary considerably e.g. the addition of some B vitamins, p-amino-benzoic acid or folic acid are required singly or in conjunction for the growth of most representatives of this family (Hutner, 1946,1950,1961). Rm. vanniellii does not require any added vitamins nor does the addition of these stimulate growth (Duchow and Douglas, 1949). Similarly a comprehensive study of the vitamins and stimulants on the growth of Rhodomicrobium (Rm5) proved that none were required or beneficial

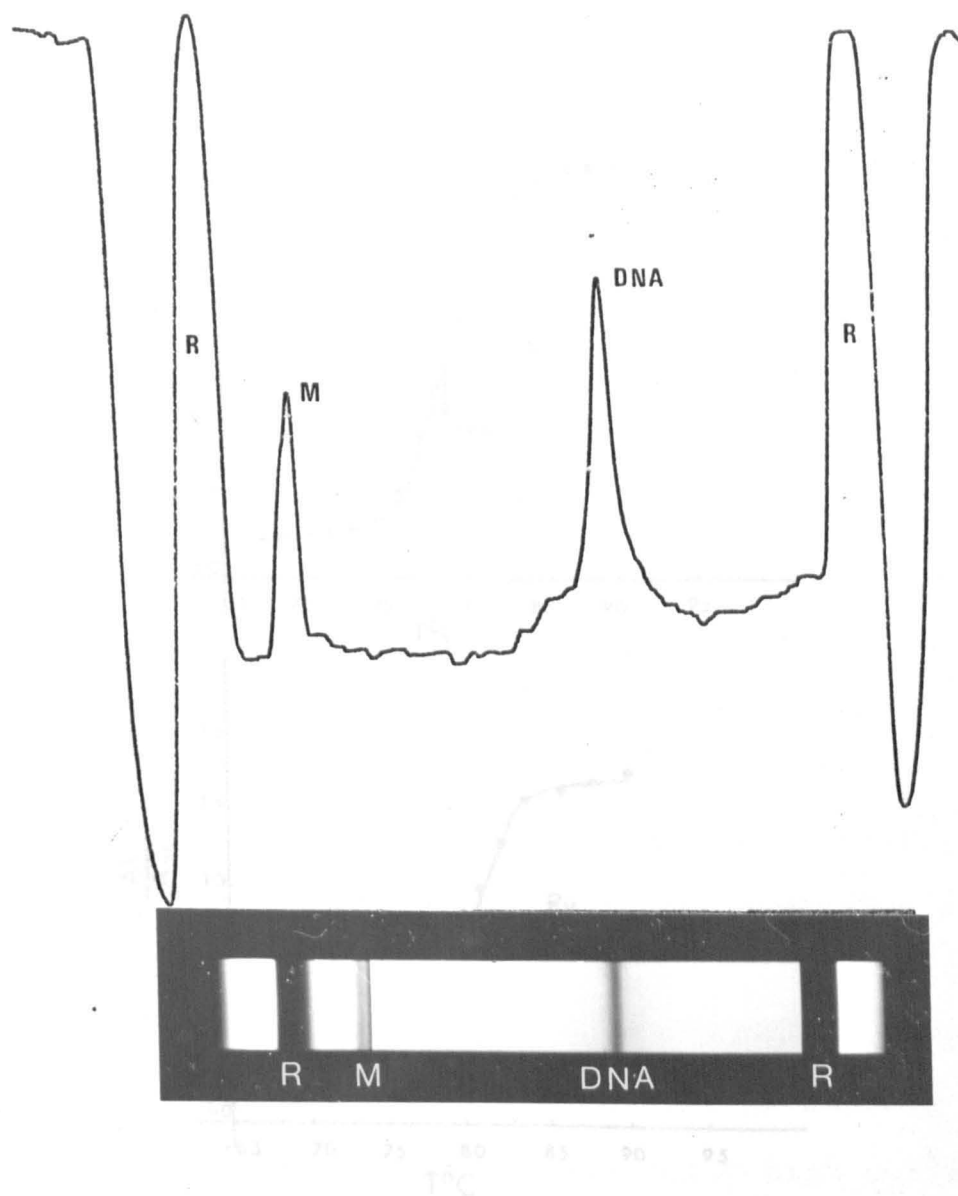


Figure 3.12

Analytical neutral CsCl buoyant density centrifugation of 1 μ g of both Rhodomicrobium(km5) and Rm. vanniellii DNA.

and Rm. vanniellii DNA suspended in 0.1 SSC.

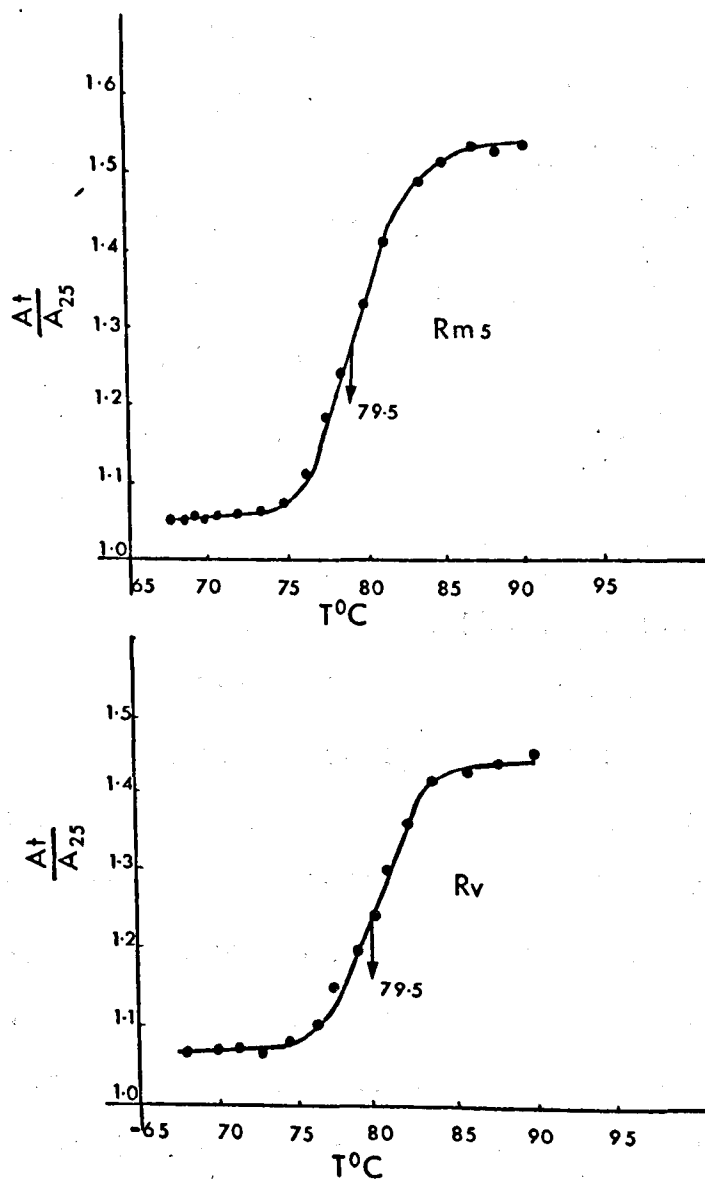


Figure 3.13

Temperature profile analysis of Rhodomicrobium(Rm5) and Rm. vannielii DNA suspended in 0.1 SSC.

(b) N₂ Sources.

Table 3.3 illustrates the growth of Rm. vannielii and Rhodomicrobium(Rm5) on a variety of nitrogen sources.

Potassium nitrate was not assimilated and urea to only a very limited extent. Although casamino acids supported reasonable growth this was restricted and may be interpreted as inhibition by specific amino acids (Scardovi, 1953; Coleman, 1959; Tuttle and Gest, 1960). Ammonium salts gave consistently high growth rates.

Whole cells of Rm. vannielii and Rhodomicrobium(Rm5) were grown in malate medium (SpR, section 3.2) lacking bound nitrogen but under gaseous nitrogen and tested for their nitrogenase activity by measuring the reduction of acetylene to ethylene chromatographically (Postgate, 1972). Both organisms had similar rates of conversion and grew equally well under such environmental conditions (figure 3.14). No quantification experiments were undertaken but preliminary results indicate several parameters to be important in maintaining consistently high activities eg. light intensity, concentration of cells, pH and temperature. When fixing nitrogen the growth rate was below the optimum, swarm cells were absent, but the sporulation cycle of Rhodomicrobium(Rm5) remained as normal. No morphological abnormalities were observed

(c) Carbon source utilisation.

No formal screening of particular carbon source assimilation by Rm. vannielii and Rhodomicrobium(Rm5) was undertaken except with respect to carbon sources promoting increased growth rates.

Species of purple non sulphur photosynthetic bacteria utilise alcohols and ketones, carbohydrates, compounds of

N ₂ source	growth	final pH
NH ₄ Cl	++++	7.7
Casamino acids	++	7.6
KNO ₃	-	7.0
Urea	(+)	7.0
Atmospheric N ₂	+	7.1
No added N ₂	-	7.0

Table 3.3

Growth of Rm.vannielii and Rhodomicrobium (Rm5) on a variety of N₂ sources.

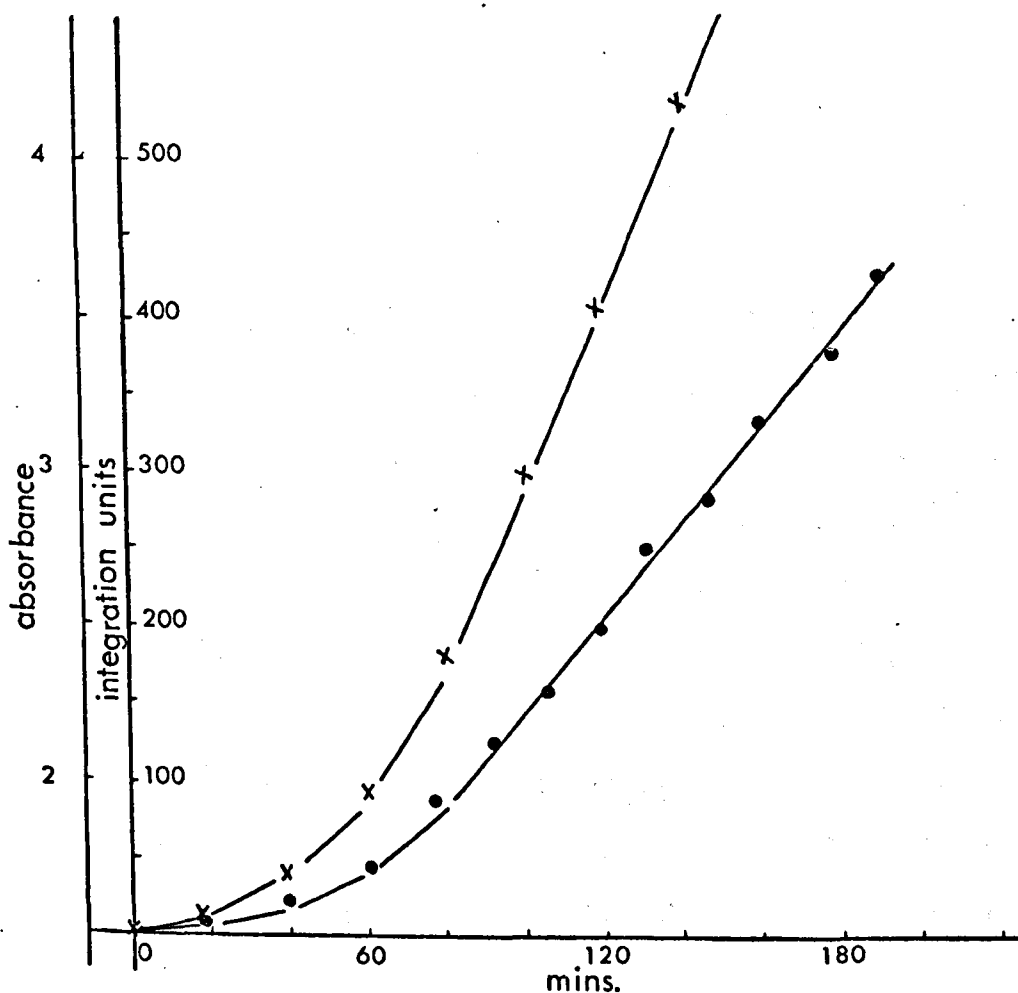


Figure 3.14

Fixation of atmospheric nitrogen by Rhodospirillum(Rm5) grown photosynthetically (1,000 lux light intensity) in malate medium (SpR) lacking a fixed nitrogen source.

x Nitrogenase activity, i.e. reduction of acetylene to ethylene determined chromatographically.

● Optical density at 540 nm.

the aromatic series and a large variety of organic acids (Kondrat'eva, 1965). Rm. vanniellii has been grown experimentally on a variety of carbon sources but primarily on two organic acids, lactate and acetate (Duchow and Douglas, 1949; Morita and Conti, 1963; Conti and Hirsch, 1965; Trentini and Starr, 1967). Trentini and Starr (1967) reported a maximum specific growth rate of 0.17 hr^{-1} at 500 ft-c on sodium lactate. Throughout this study malate (SpR, section 3.2) or malate pyruvate (PYR, section 3.2) medium was used and gave specific growth rates of 0.14 hr^{-1} and 0.175 hr^{-1} respectively at a light intensity of 2,000 lux. Substitution of the carbon source by sodium lactate or increasing the light intensity did not improve these figures.

(d) Aerobic growth in the dark.

All reports to date on Rm. vanniellii (Duchow and Douglas) indicate that this organism is an obligate anaerobic phototroph. This was found not to be so in this study, the strain provided by Prof. R. Whittenbury (which was the Duchow and Douglas isolate) and all Rhodomicrobium species obtained during the course of this study grew aerobically in the dark on SpR and PYR medium. In this respect they are similar physiologically to Rh. palustris (Rolls and Lindstran, 1967) and Rh. acidophila (Pfennig, 1969).

Three different methods were used to test for the ability of Rhodomicrobium species to grow aerobically or microaerophilically in the dark; stationary liquid cultures, agar shake tubes and agar plates. All gave positive results but required long incubation periods; from three to five weeks. Colonies grown in the dark on aerobic agar plates were heavily pigmented (red brown), irregular and

characteristically very tough, a property imparted by the chain mode of growth (plate 3.5).

(e) Cytochromes.

The published literature on the Athiorhodaceae cytochrome complement is of interest in the context of this thesis with regard to aerobic growth of Rhodomicrobium in the dark. Morita and Conti (1963) characterised the cytochromes of Rm. vannielii (considered to be an obligate anaerobic photoheterotroph) primarily because of its ability to take up oxygen in cell suspension and cell free extracts (Morita and Conti, 1962), an ambivalent type of behaviour for a so-called strict anaerobe. They showed the presence of one b type, two c type cytochromes and a carbon monoxide binding haemoprotein which would appear to be very similar to the terminal oxidase, cytochrome o (Peterson, 1970). A study here of cell extracts of Rm. vannielii (Duchow and Douglas) and Rhodomicrobium (Rm5), examined by the method of Rieske (1967), showed the presence of two c type cytochromes and a CO sensitive cytochrome. There was apparently no b type cytochrome but this may have been masked by the unusually high cytochrome c content.

Table 3.4 lists the cytochromes characterised in the literature for Rm. vannielii (Morita and Conti, 1963) and Rh. palustris (Bartsch, 1971) in addition to those characterised here for Rhodomicrobium (Rm5).

Studies on the cytochrome content of non-sulphur purple bacteria grown aerobically in the dark (Kikuchi, 1965,) have lead to the conclusion that a cytochrome oxidase with unusual properties functions as a terminal oxidase in the dark respiration process. However,

Plate 3.5

Rm. vanniellii and Rhodomicrobium (Rm5) grown
aerobically at 30°C in the dark on PYR medium.

Table 3.4

Cytochromes characterised for Rm. vanniellii, Rhodomicrobium (Rm5) and Rh. palustris.

Organism	Cytochrome	max.	reference
<u>Rm.vanniellii</u> (Duchow and Douglas)	c	550,522,414	Morita
	c	553,521,423	and
	b	563,530,423	Conti
	CO binding		(1963)
	hemoprotein-cytochrome O?	570,538-543,414	
<u>Rhodomicrobium Rm5</u>	c	550,520	
	c	555,520	this thesis
	CO binding		
	hemoprotein-cytochrome O?	570,526,413	
<u>Rh. palustris</u>	c ₂	551.5,418	
	cc ₁	552,426	Bartsch
	c ₃	551.5,418.5	(1971)
	c ₅₅₄	554,418.5	
	c	551.5,420	
	b	559,425.5	

there are close similarities between the majority of cytochromes formed irrespective of the growth conditions i.e. photoorganotrophic or chemoorganotrophic. Although analysis of aerobic dark cell cultures of Rhodomicrobium were not undertaken, a close relationship was found between the cytochrome content of anaerobically grown cells and that of similarly grown cells of Rh. palustris, suggestive of a close physiological relationship between these species.

The findings of Morita and Conti (1962) and the results obtained here concerning the cytochrome characteristics of Rhodomicrobium are consistent with the notion that these bacteria are able to grow aerobically in the dark and are therefore similar in this aspect of their physiology to other non-sulphur purple bacteria.

As far as can be judged from the morphological and physiological characteristics examined and described above, Rm. vanniellii (Duchow and Douglas) and Rhodomicrobium (Rm5) are strains of the same species. In consequence only the developmental growth pattern of Rhodomicrobium (Rm5) was studied in detail.

D) Growth and development of Rhodomicrobium(Rm5) in Batch culture.

Rhodomicrobium, grown in batch culture, is characterised during the specific growth phases by changes in morphology and functional cell expression (figure 3.8). The individual cellular entities, chain forming cells (Duchow and Douglas, 1949), motile cells (Douglas and Wolfe, 1959) and resting cells (Gorlenko, 1969) have received only very superficial characterisation. There is no substantiated information on the derivation, developmental pattern or integrated cultural characteristics of any of these cell types.

(i) Lag and early exponential phase growth.

When a vegetative culture of Rhodomicrobium(Rm5) is inoculated into fresh medium (SpR or PYR medium, section 3.2) the early exponential growth phase consists of typical chains of cells (plate 3.6) the ovoid mature cells of which are 2-2.5 μ in length and 1 μ in diameter. Although the interconnecting filament may vary in length from 3 μ to upwards of 25 μ , depending on the growth rate, the diameter is constantly 0.2 μ .

Reproduction is by terminal swelling of the filament i.e. bud formation, the daughter cell upon maturity remaining attached but isolated by a filament 'plug' or constriction from the mother cell (Duchow and Douglas, 1949). There is no information in the literature pertaining to the fate of either cell. Studies on slide culture of the growth and development of Rhodomicrobium allows some of the complexities of the growth pattern to be unravelled and revealed that certain steps were obligatory for growth and reproduction, allowing each cell in a chain to be sequentially identified with regard to formation.

Slide cultures of SpR medium were prepared (section 2.2) inoculated with a photosynthetic, exponentially growing Rhodomicrobium (Rm5) culture and followed microscopically through several generations (light energy being supplied by the microscope illumination system). The generation time, from initiation of filament synthesis through bud formation to maturity, was 4½-5 hrs, maturity being assessed as the time of observed filament synthesis from the daughter cell. The estimation of the generation time from such microcultures, however, varied considerably due to the inconsistency of

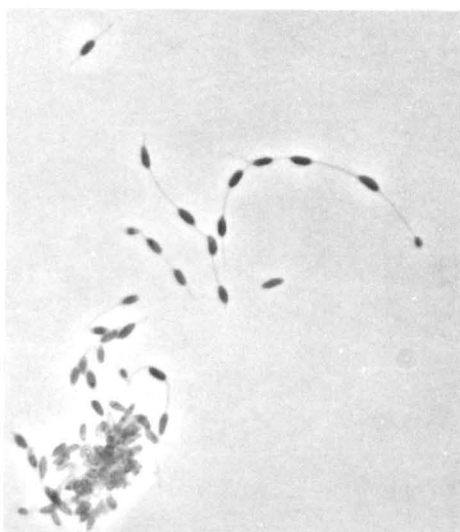


Plate 3.6

Phase contrast photomicrographs of vegetative chain formation in Rhodomicrobium (Rm5). (x1100)

her cell is dependant on the growth rate i.e. filament length, as shown in plate 3.9. The development prior to these cellular configurations, as formulated from slide cultures, is shown diagrammatically.

Having formed a daughter cell and in consequence 'locked' itself in a chain configuration, the mother cell eg. m2, plate 3.9, can only produce a second and subsequent daughter cells by the synthesis of branches from the main or original filament. Such dependance on branching is illustrated in the viable system, plate 3.10, and in the electron micrograph, plate 3.9, which also illustrates the continuity of the mother cell with the branch filament. Secondary filaments, excluding initial bidirectional growth potential of swarm cells, do not arise directly from the mother cell body, any cells doing so are abortive.

the residual oxygen concentration. The sequential development steps are illustrated by several cells in plate 3.10, cell clusters a, b and c being analysed separately and diagrammatically summarised in figures 3.15,16,17.

Electron microscopic examination of the cell systems reveals that mature individual cells are isolated, not only physically but most probably physiologically by filament 'constrictions' or 'plugs' (plate 3.7,3.8). The positioning of these strongly suggests that their synthesis is under the control of, and specified by the daughter cell. The contention is that upon maturity i.e. physiological independence, the daughter cell isolates itself from the mother. This is supported by the distance of the constriction from the daughter cell being invariably constant whereas the distance from the mother cell is dependant on the growth rate i.e. filament length, as shown in plate 3.9. The development prior to these cellular configurations, as formulated from slide cultures, is shown diagrammatically.

Having formed a daughter cell and in consequence 'locked' itself in a chain configuration, the mother cell eg. m2, plate 3.9, can only produce a second and subsequent daughter cells by the synthesis of branches from the main or original filament. Such dependance on branching is illustrated in the viable system, plate 3.10, and in the electron micrograph, plate 3.9, which also illustrates the continuity of the mother cell with the branch filament. Secondary filaments, excluding initial bidirectional growth potential of swarm cells, do not arise directly from the mother cell body, any cells doing so are abortive.

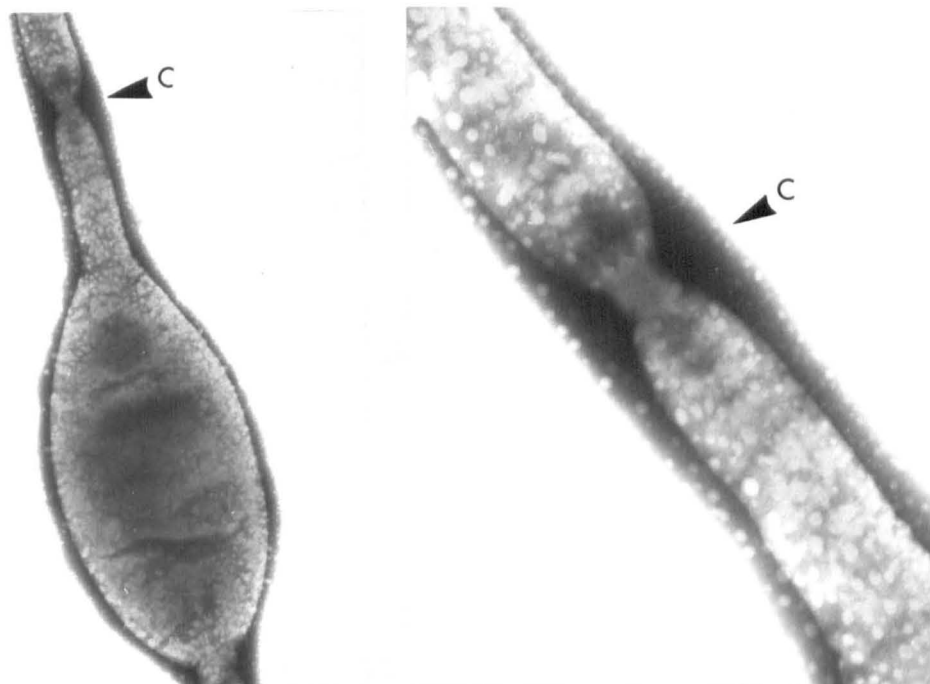


Plate 3.7

Aqueous uranyl acetate (0.5% w/v) negatively stained preparation of a Rhodomicrobium(Rm5) mature vegetative cell showing filament constriction(c) or 'plug'. (x20,000 and 60,000).

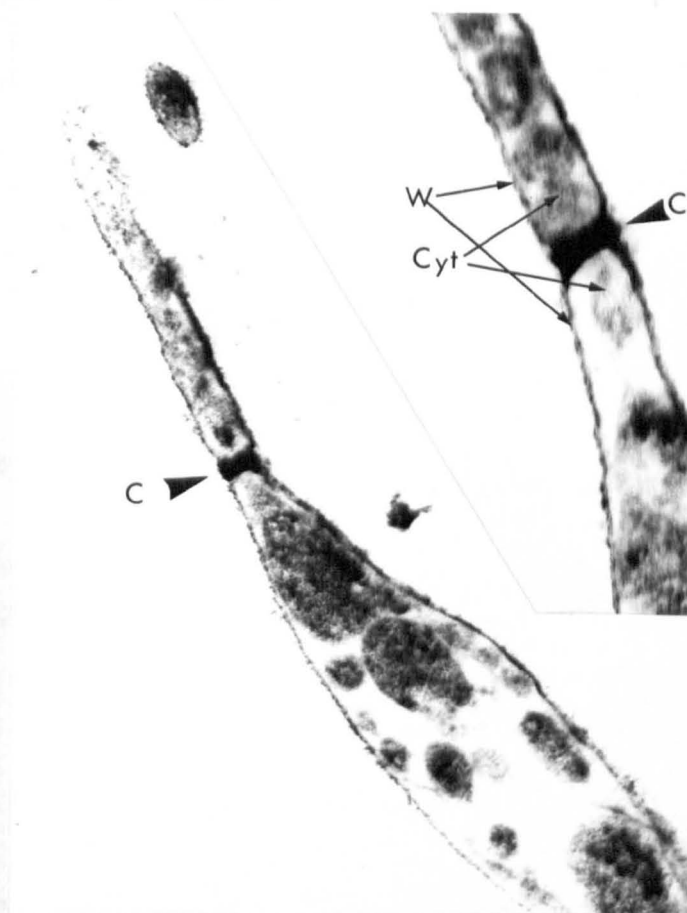
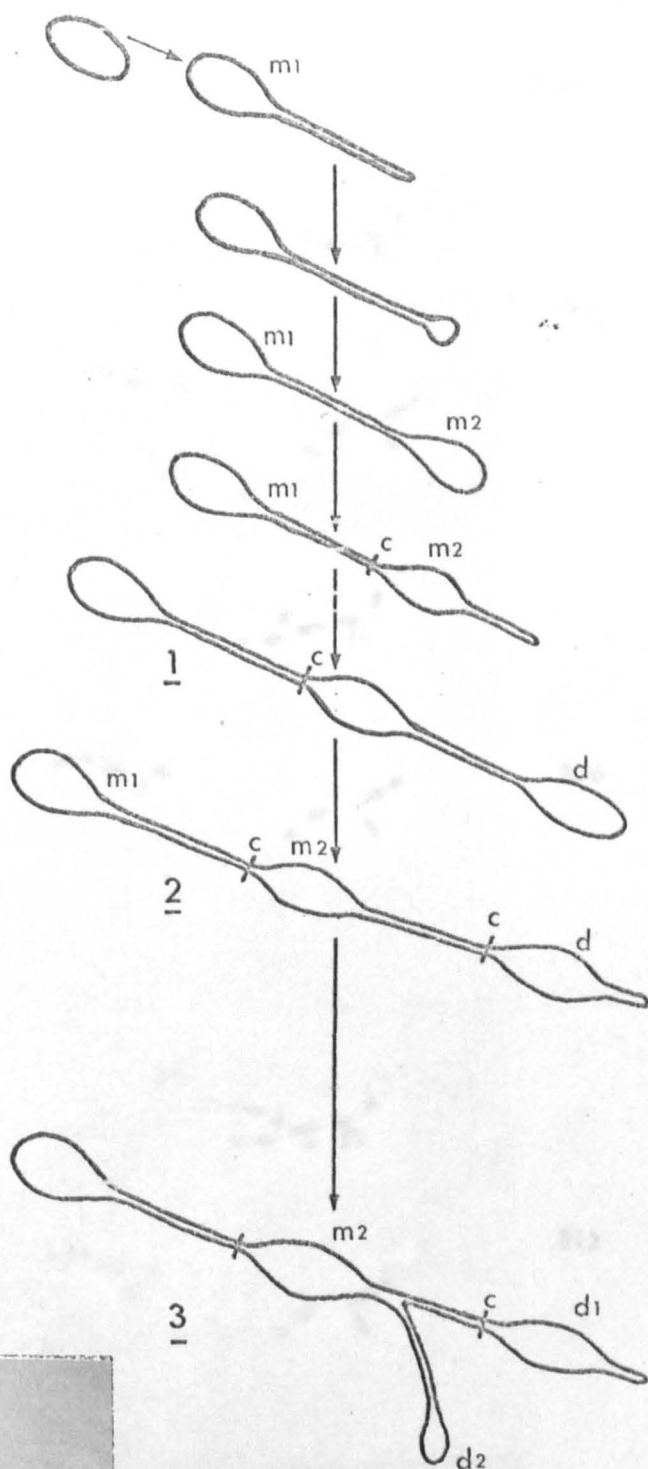
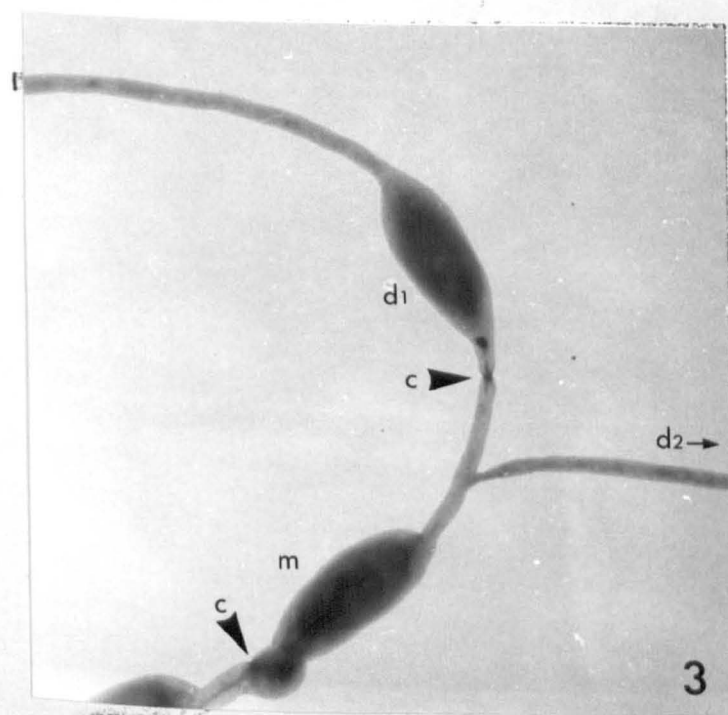
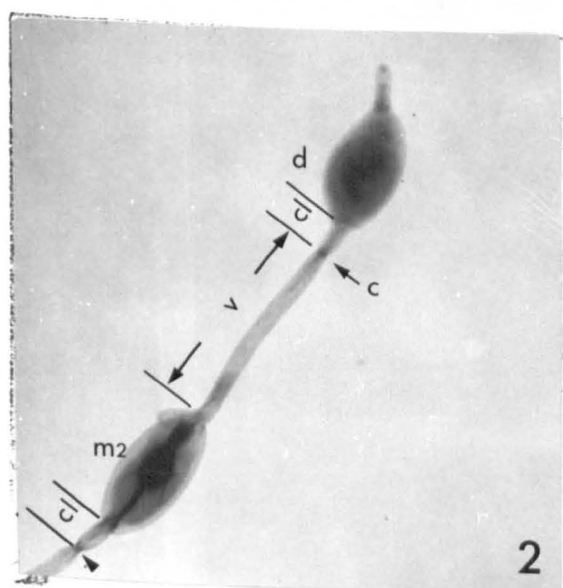
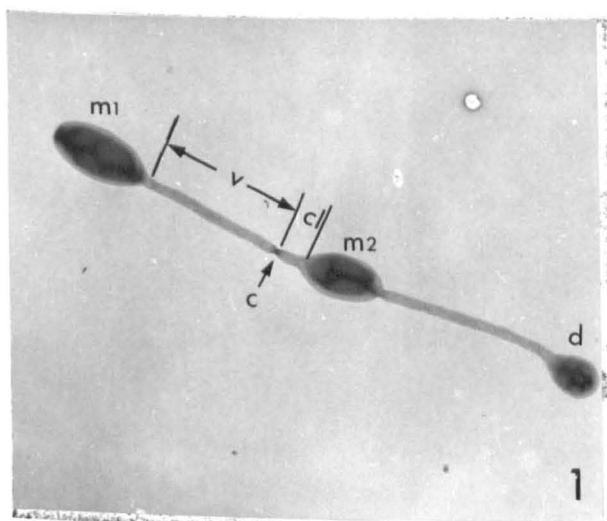


Plate 3.8

Thin section of Rhodomicrobium(Rm5) vegetative cell showing solidarity of constriction (c). (x 20,000 and 40,000)



Electron micrographs (light metal shadow) showing sequence of growth and formation of filament constrictions (c) upon cell maturity with constant spacing (c_1) from the daughter cell (d) and variable positioning relative to mother cell (m_1 or m_2).

(x 8,000)

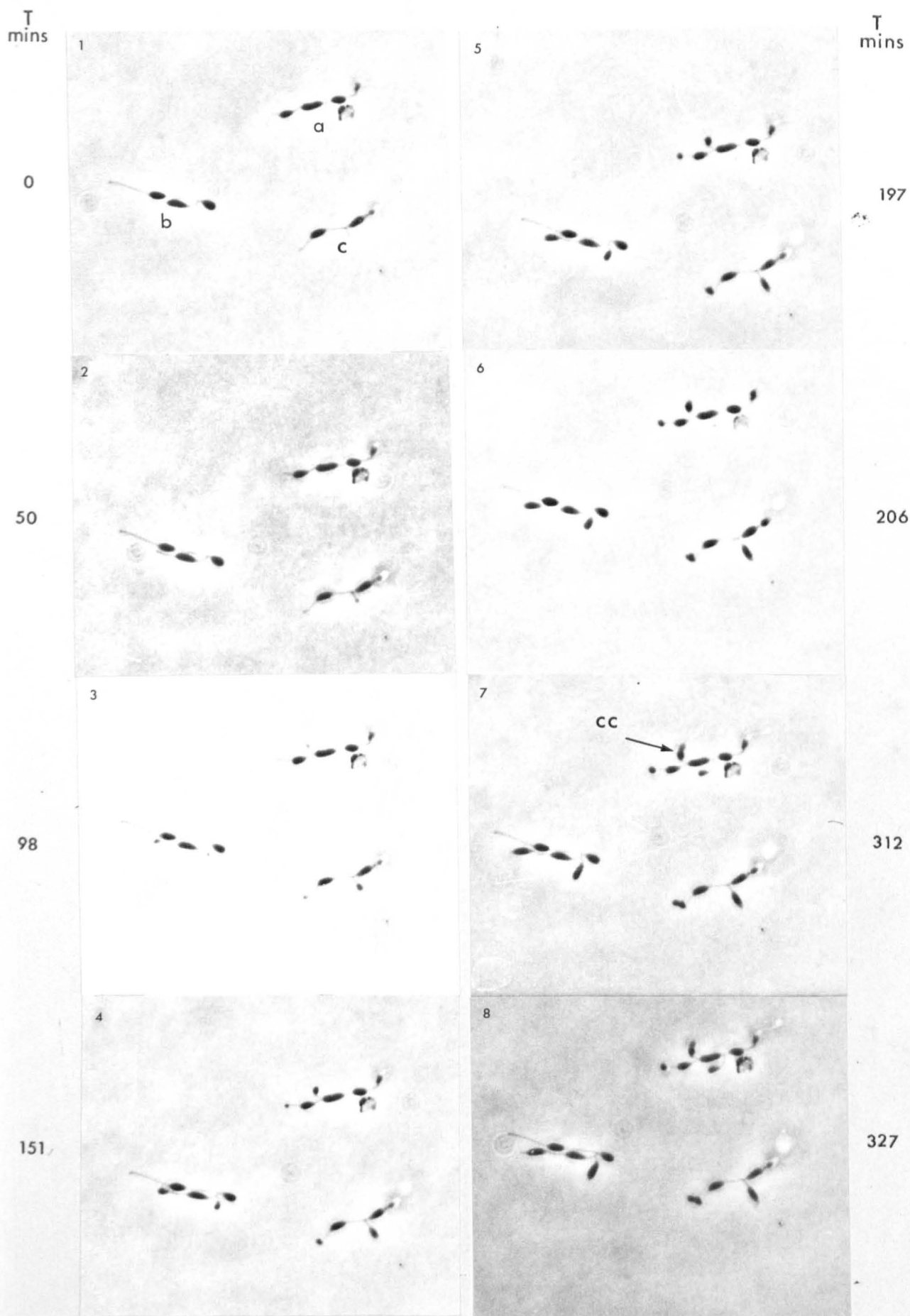


Plate 3.10

Sequential phase contrast photomicrographs of *Rhodomicrobium* (Rm5) vegetative cell growth. (x1100)

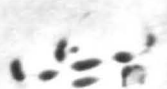
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414



10

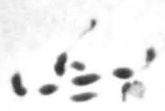


446

Plate 3.10

(continued)

11



488



12



528



13



637



Figures 3.15, 3.16, 3.17.

Diagrammatic representation of the growth pattern of individual cell clusters illustrated in plate 3.10, showing the derivative cell for each daughter and sites of constriction formation.

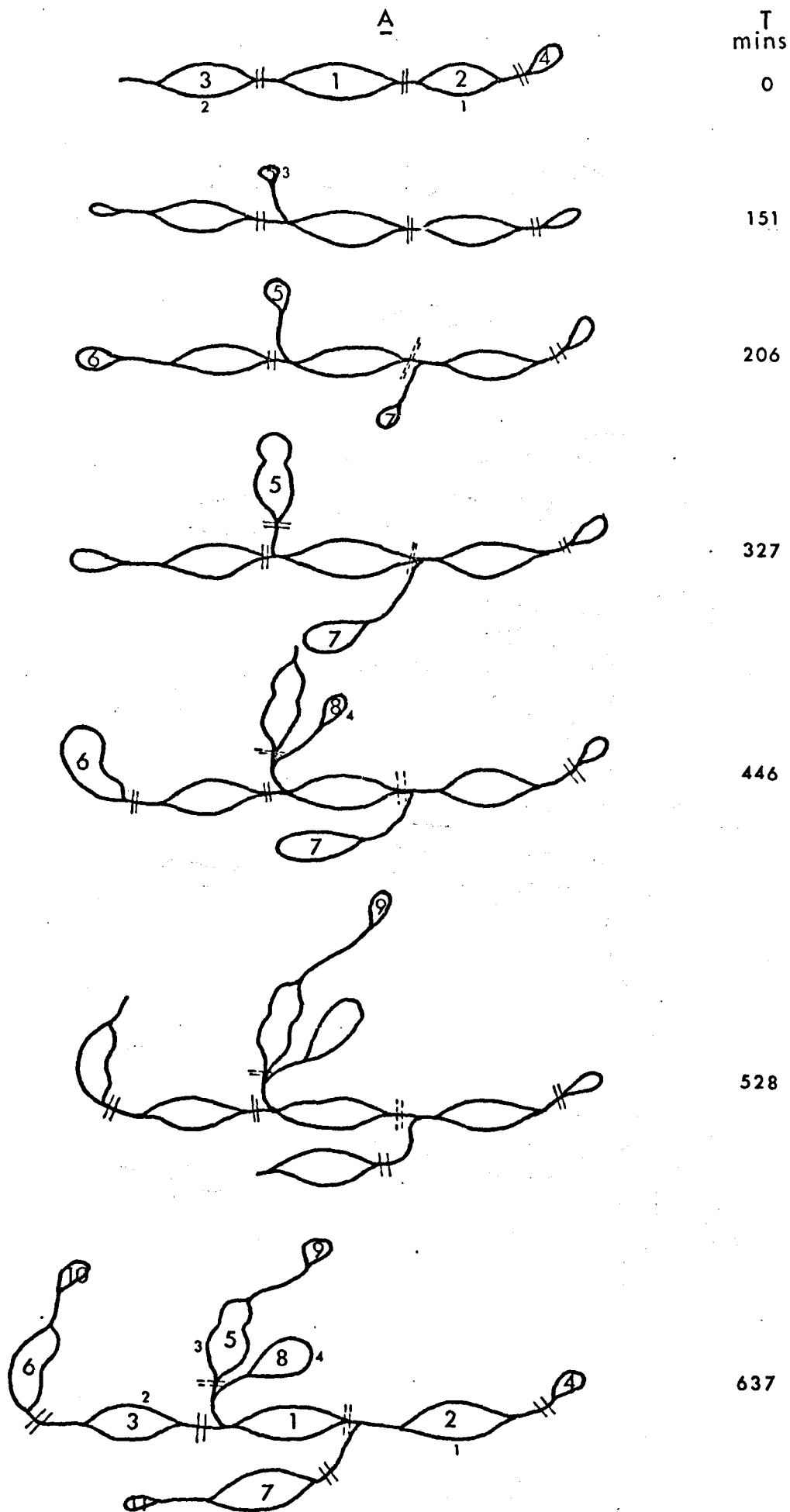


Figure 3.15

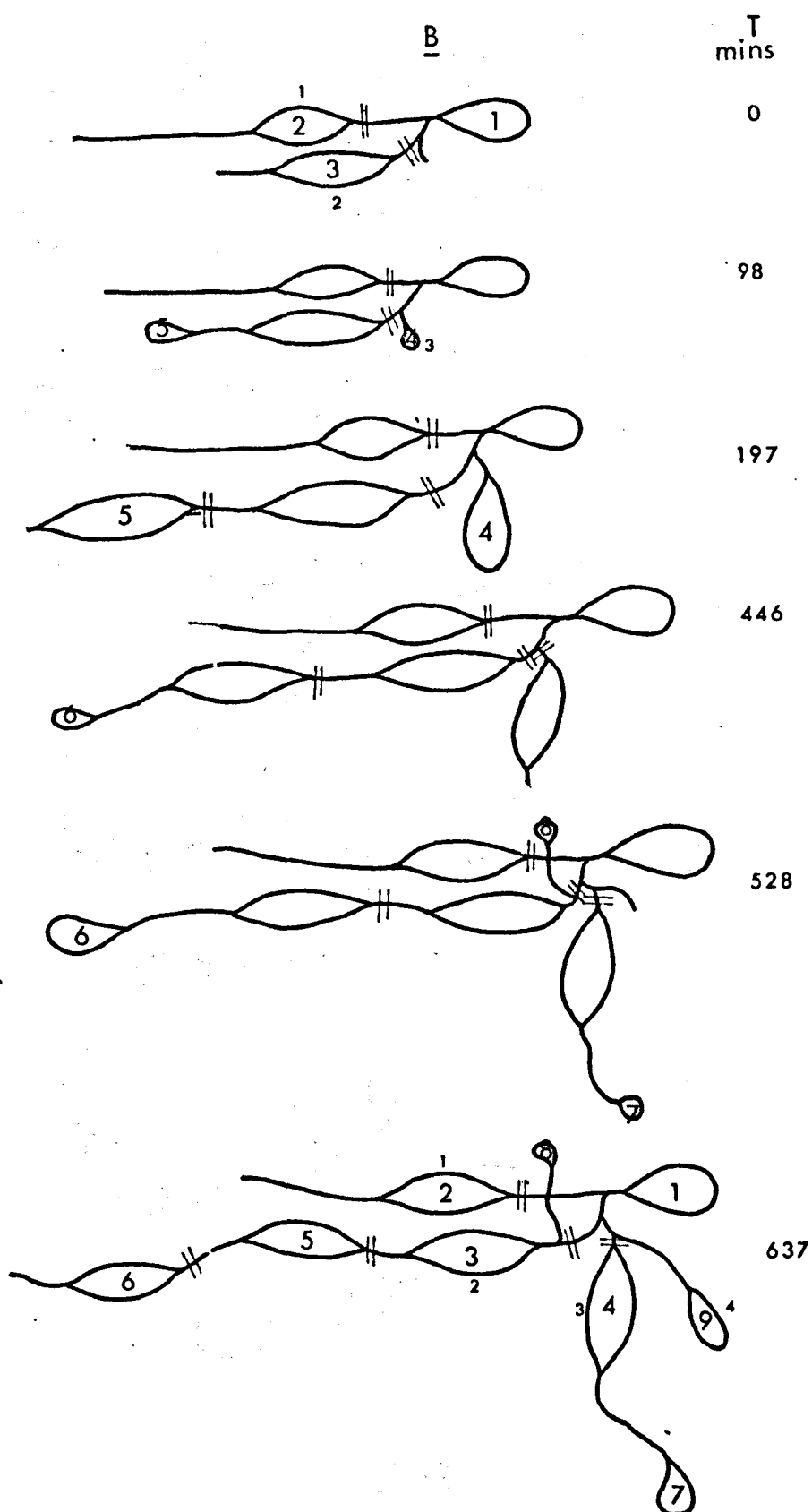


Figure 3.16

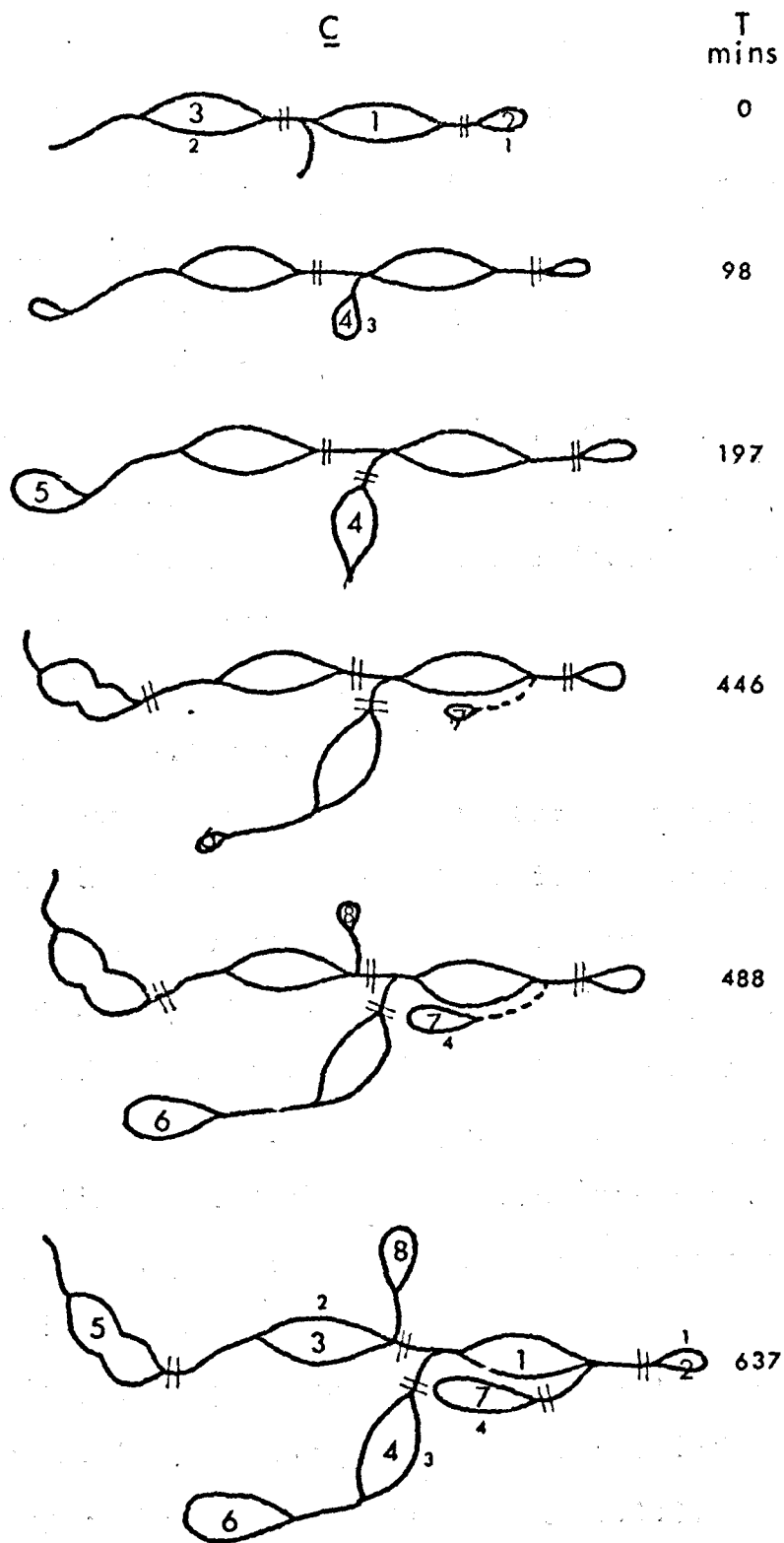


Figure 3.17

Detailed sequential studies of slide culture growth has allowed formulation and substantiation of the following 'rules' which are strictly observed by developing Rhodomicrobium cells:

1. Any cell has the potential to form one and only one cell at a time.
2. Upon physiological maturation, cells are isolated by characteristic 'plugs' whose synthesis appears to be under the control of the developing daughter-not the mother cell.
3. Branch formation occurs on the most recently synthesised filament.
4. Filament synthesis is obligatory for cell reproduction.

Adhering to these parameters it became apparent that any one mother cell can give rise to a maximum of four daughter cells (plate 3.10, figures 3.15,16,17). Although variation of cultural conditions will determine whether 1, 2, 3 or 4 siblings are formed per mother cell, no more than 4 siblings per mother cell were ever recorded. The physiological implications arising from this observation will be discussed later.

This growth pattern is further complicated by the presence, although infrequent, of a constricted cellular morphology - the "double cell" (plate 3.11). These cells are formed by the normal budding process (plate 3.10,7) with a generation time of 5 hrs. They remain viable yielding 'normal' daughter cells according to the formula already described. The physiological and ultrastructural characteristics of this cell type were undetermined.



(a) Swarm cell formation.

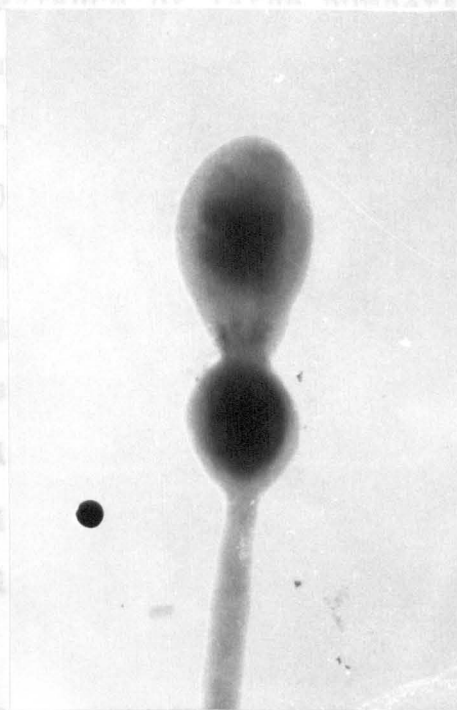


Plate 3.11

Light metal shadow of constricted cellular morphology characteristic of a few Rhodomicrobium(Rm5) vegetative cells grown in chain formation. (x 12,000).

Growth and reproduction in such a stringent pattern leads to several intriguing questions. How does the cell instigate secondary filament formation in an established system? What parameters determine branch formation? What relationship does the DNA have with the branch point? Where and when does DNA replication occur? Why is a maximum of only four daughter cells formed? What is the physiological 'state' of the mother cell after synthesis of the fourth daughter? Does the non reproductive, isolated mother cell perform unique and characteristic biochemical functions?

(ii) Exponential growth phase.

(a) Swarm cell formation.

The exponential growth phase is characterised primarily by the appearance of large numbers of peritrichously flagellated swarm cells which are formed by terminal budding from either main or branch filaments (plate 3.12). Douglas and Wolfe (1959) first observed Rhodomicrobium swarm cells and described their release from the filament as being "through cell agitation caused by flagellar activity". This is illustrated in plate 3.13 by cell b, which in (1) is actively motile and attached but is subsequently released(2). In contrast cell a (plate 3.13,1-4) although motile initially (2) remains attached and becomes part of the chain i.e. possession of flagella does not automatically mean that the cell will be released. The question therefore arises "are all daughter cells flagellated?" The answer is no. With exponential chain formation and swarm cell daughter development, neither are flagellated (as determined by electron microscopy).

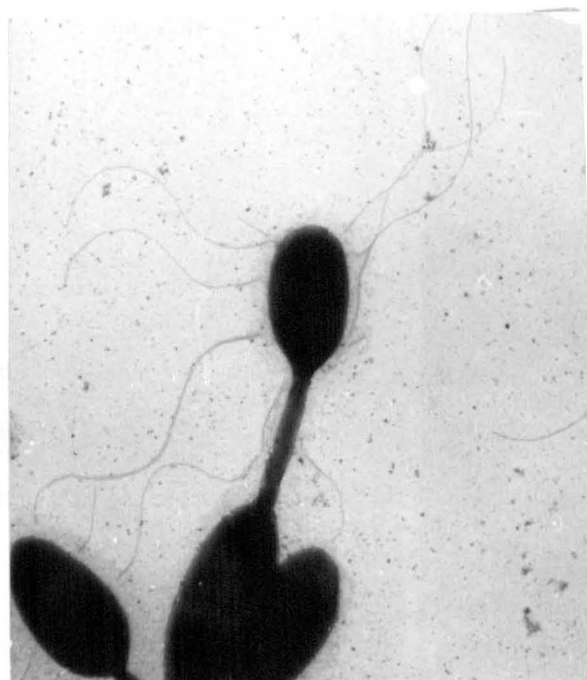
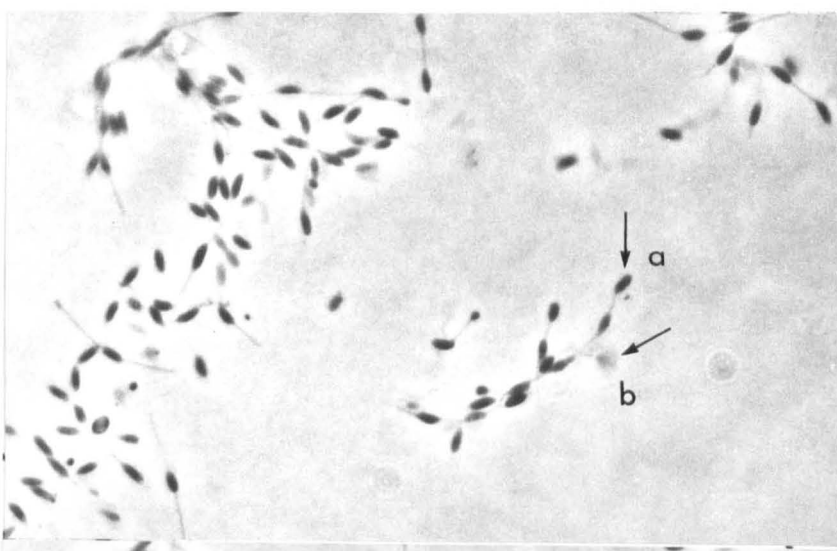


Plate 3.12

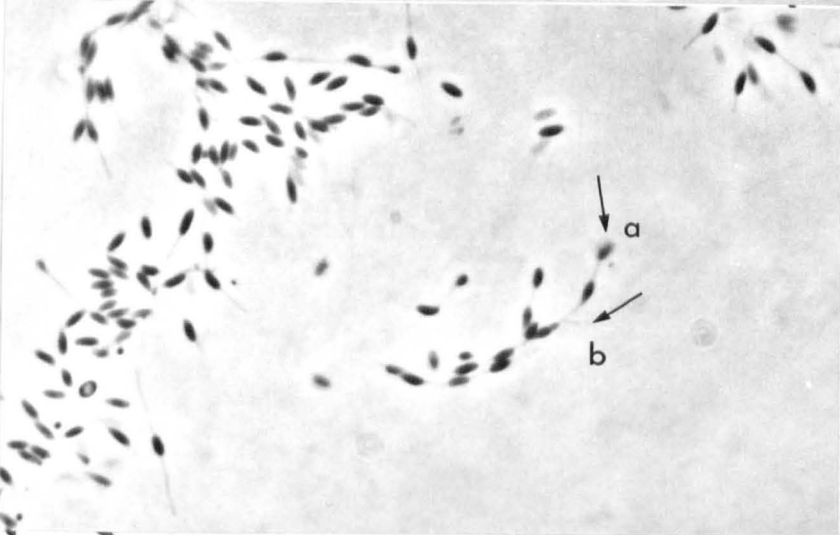
Aqueous uranyl acetate (0.5% w/v) negative stain
of swarm cell formation. (x 12,000)

Plate 3.13

Phase contrast photomicrographs of slide cultures
of Rhodocyclidium (Rm5) showing swarm cell formation
(a and b) and flagellar aggregation (s). (x 1100).



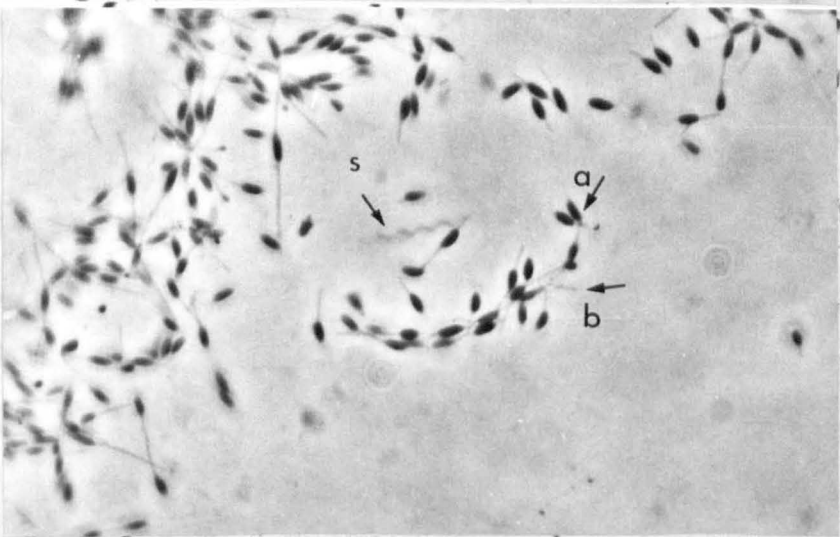
1



2



3



4

When released from the filament, flagellated daughter cells do not carry 'scars' or remnants of filament which therefore brings into focus the fact that there are two division mechanisms, i.e. a) 'plug' or 'contriction' formation as seen in chain formation and b) swarm cell release; the latter is synonymous with binary fission and corresponding to daughter cell release in Hyphomicrobium and Caulobacter. Additionally after cell release in plate 3.13, cell b, there is an increase in filament length, an observation which will be brought into context later.

(b) Swarm cell development.

The first step observed in the subsequent growth of the swarm cell is the synchronous shedding of the flagella which is concomitant with a slight change in cell shape and most probably cell wall structure since such cells bear no observed remnants of flagellar attachment (plate 3.15, 1 and 2). Subsequent filament development can follow one of two patterns:

- a) single filament formation from one pole (plate 3.14 A)
- b) double filament formation from both poles of the cell (plate 3.14 B).

Further development of either cell type is, however, identical. The cell is capable of bud formation in only one direction at a time (plate 3.14 A and B). Upon completion of the first daughter cell i.e. formation of the plug (plate 3.15, 4), the double filamented cell (plate 3.14, B) develops the second daughter cell on the preformed filament while the single filamented cell forms a branch point, (plate 3.14, A) from the main filament. These cells are subject to the

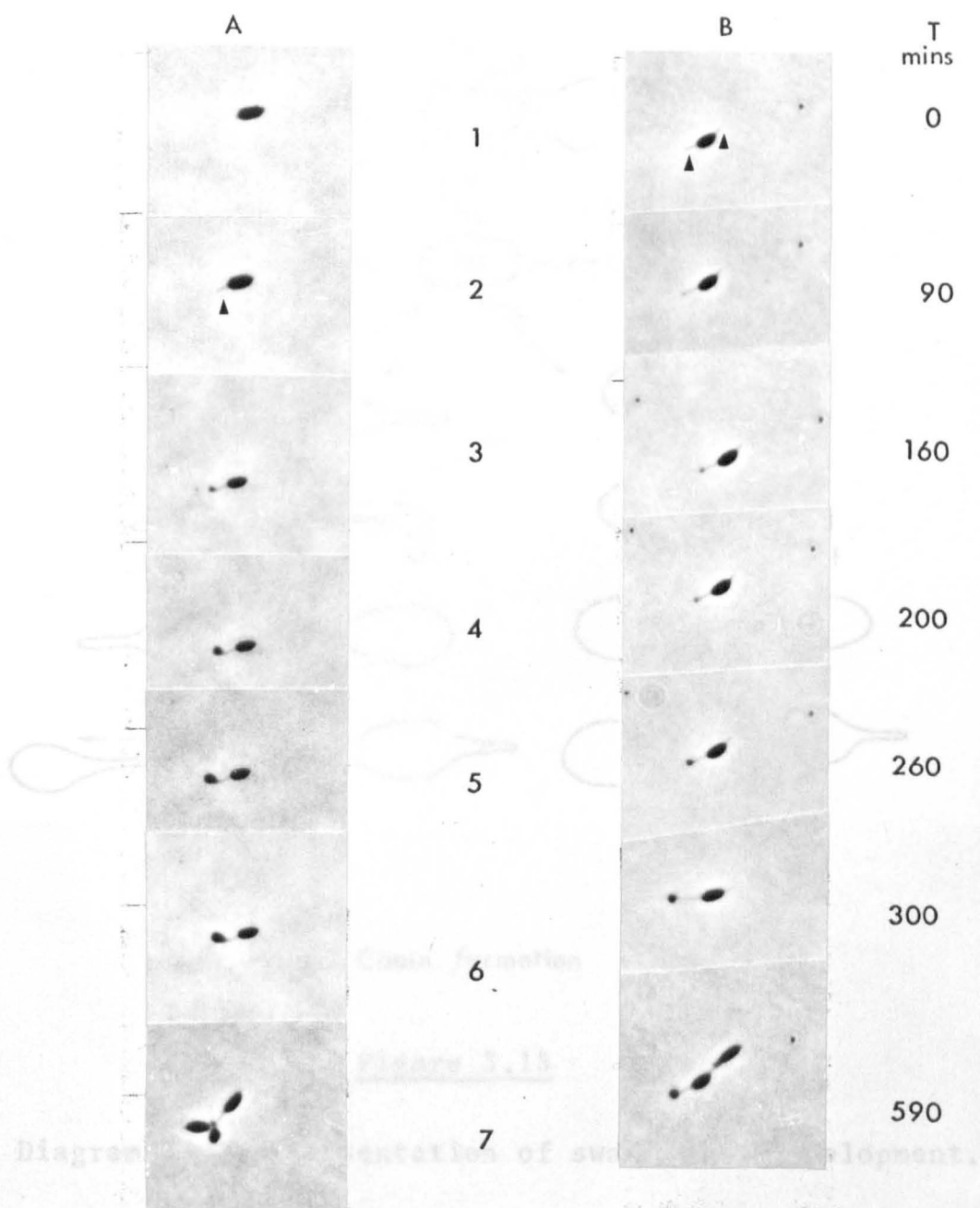
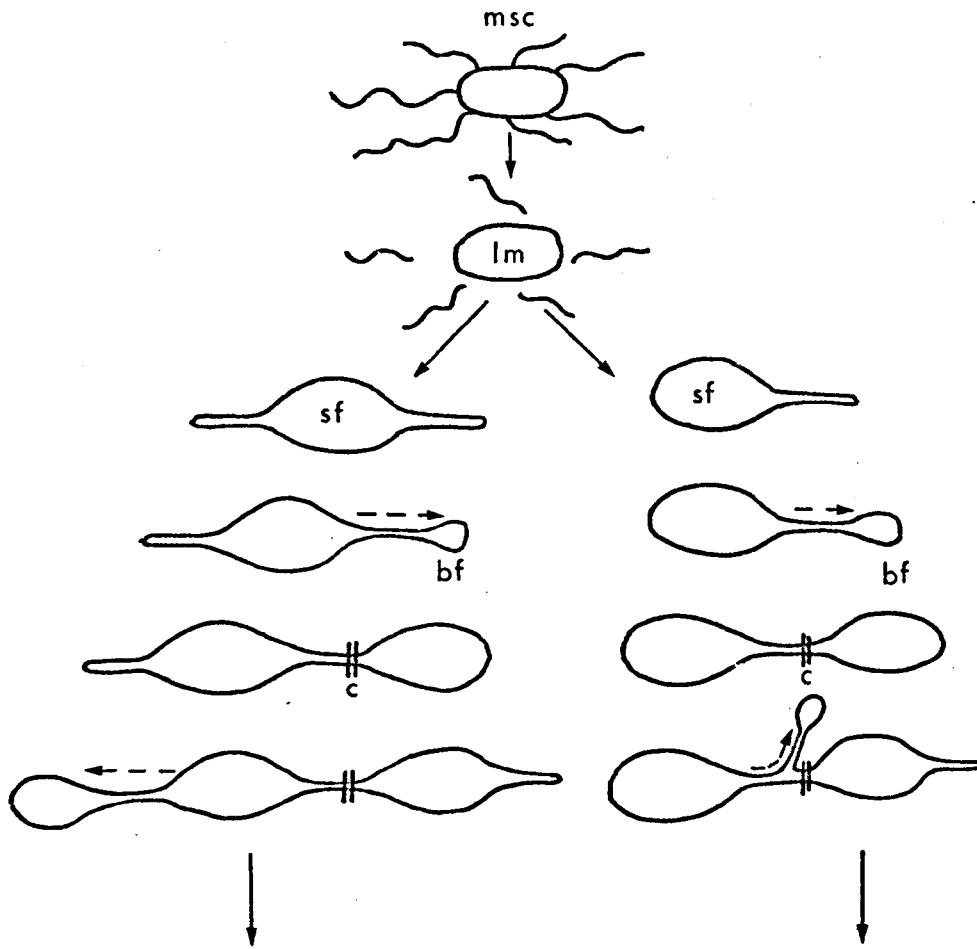


Plate 3.14

Plate 3.14

Gold Swarm cell growth and replication on slide culture followed by phase contrast photomicrography. (x1100)

1. Flagellated swarm cell (x 12,000)
2. Non flagellated cell (x 12,000)
3. (A and B) Filament formation (x 12,000)
4. Daughter cell and filament constriction (x 12,000).



Chain Formation

Figure 3.18

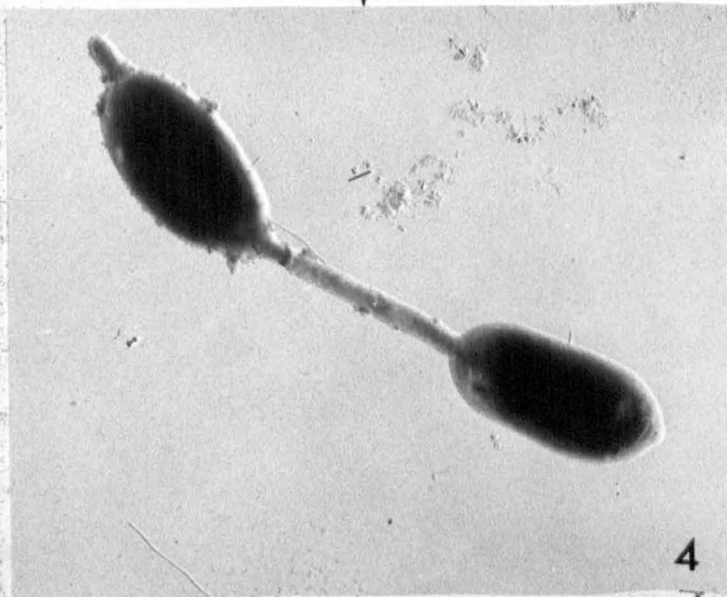
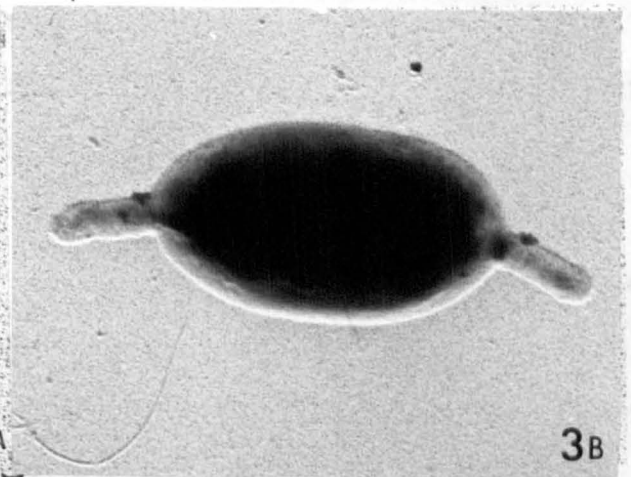
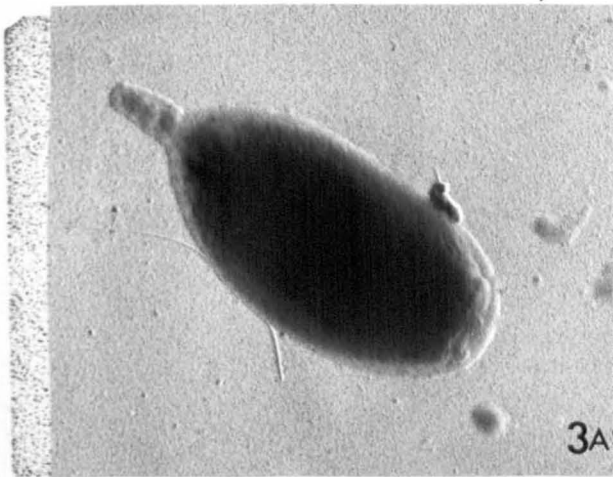
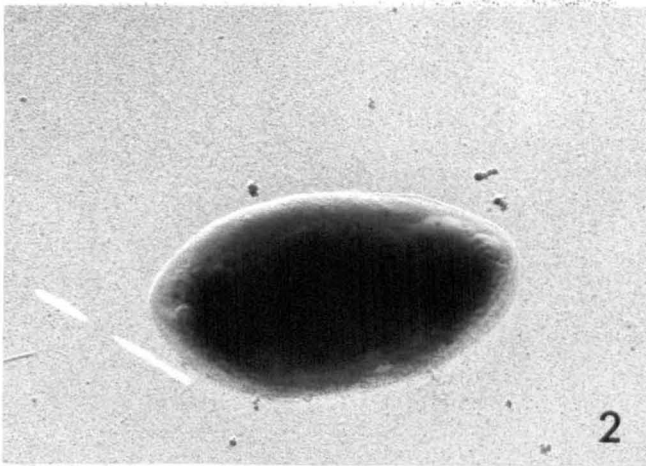
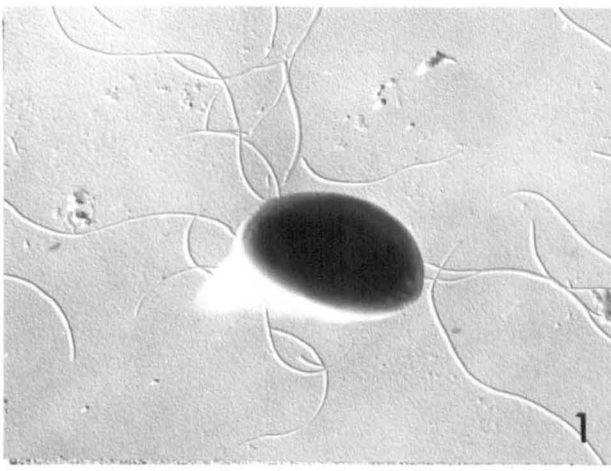
Diagrammatic representation of swarm cell development.

- msc, motile swarm cell
- lm, loss of motility
- sf, stalk formation
- bf, bud formation
- c, constriction

Plate 3.18

Gold palladium shadowed electron micrographs showing stages in swarm cell development.

1. Flagellated swarm cell (x 12,000)
2. Non flagellated cell (x 18,000)
3. (A and B) Filament formation (x 18,000)
4. Daughter cell and filament constriction (x 15,000).



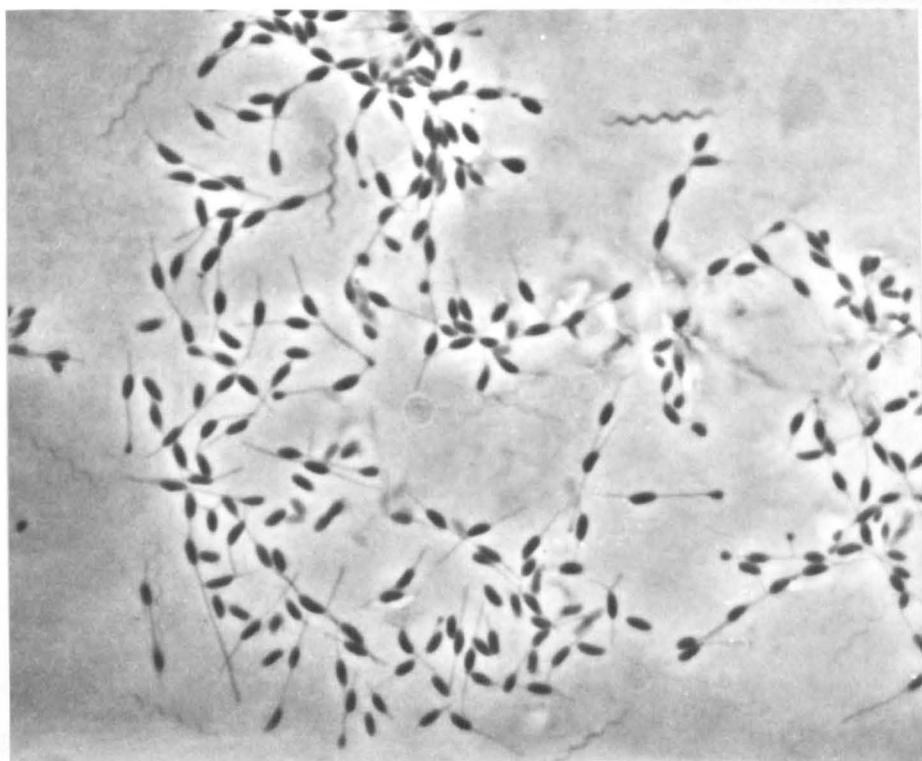
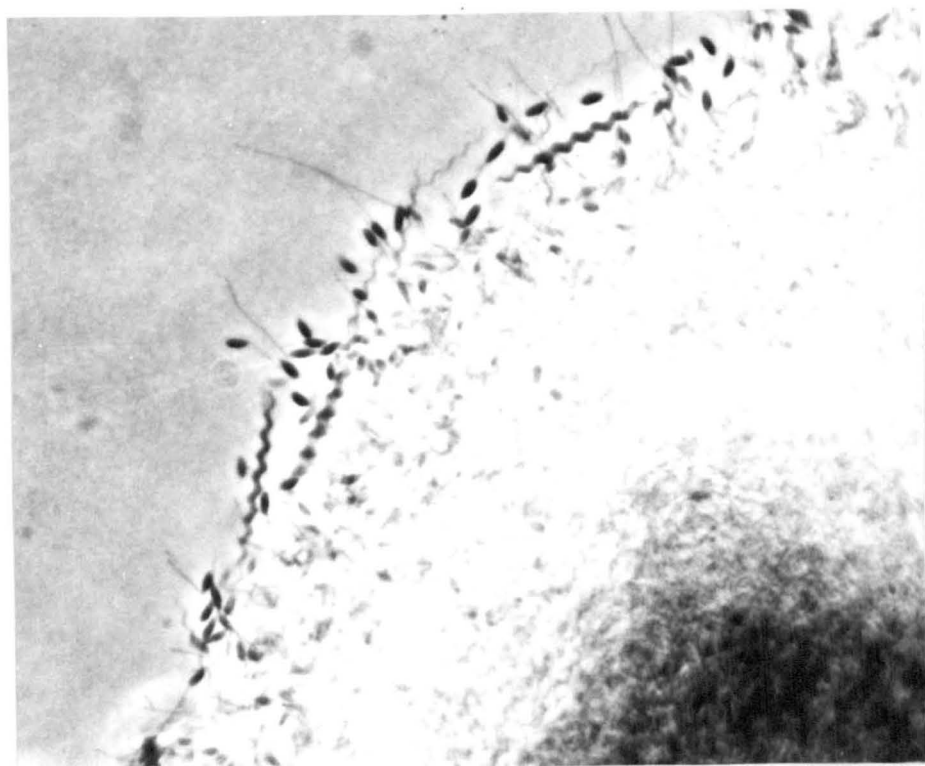
same replication restrictions and patterns as were described earlier for chain formation which in the majority of instances can be identified as originating from single swarm cells in the sequential manner described (figure 3.18).

(c) Flagella shedding.

When actively growing as slide cultures which allow dispersal of swarm cells i.e. 'wet', provided the cell concentration is high, unique and characteristic spirillar bodies appear after several hours incubation (plate 3.13, 3.16,17). These structures are rigid, relatively constant in length and wave pattern but vary considerably in diameter. They are non-viable, 'materialising' by progressive thickening (plate 3.13). They are flagellar aggregates. Motile swarm cells on differentiating i.e. filament formation, shed their flagella which in sufficient concentration, aggregate in a manner characteristic of their inherent wave pattern. The peritrichous flagellation of the swarm cell, the number of cells formed in the exponential phase (liquid culture) and the shedding phenomena enables considerable quantities of pure flagella to be isolated simply by centrifugation and, or ammonium sulphate precipitation, the purity of the preparation being assessed by polyacrylamide (6% w/v) gel electrophoresis and electron microscopy(plates 3.18,3.19).

(iii) Stationary phase.

A substantial increase in pH values (7.4-8.3) concomitant with exhaustion of nutrients is conducive to the formation of considerable numbers of angular cellular structures which in their optical properties, reduced permeability to dyes and increased resistance characteristics correspond



Plates 3.16 and 3.17

Phase contrast photomicrographs of Rhodomicrobium (Rm5) grown on slide culture showing characteristic spirillar formation. (x 1250)

1. Rhodomicrobium, Flagellin.

2. Bovine serum albumin, Flagellin.

3. Flagellin.

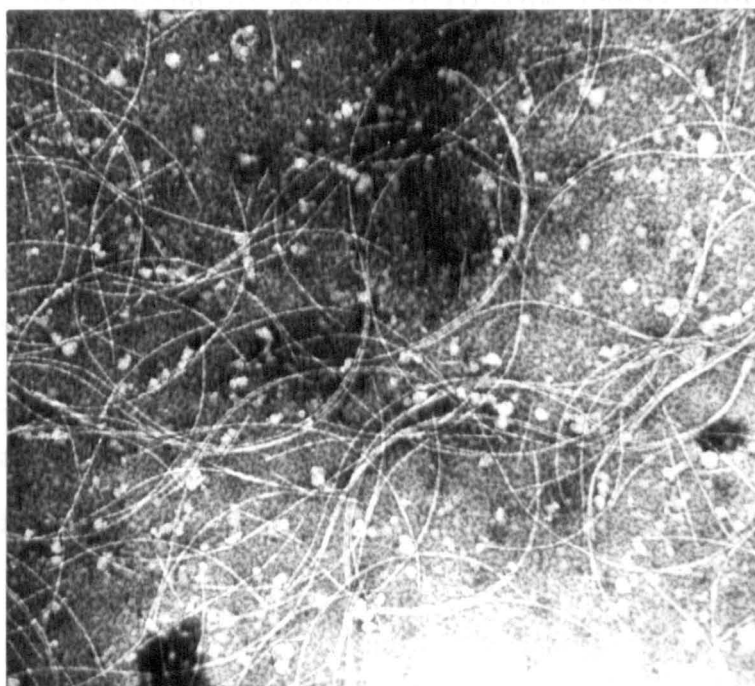


Plate 3.18

Potassium phosphotungstic acid (1% w/v) negative stain of Rhodomicrobium (Rm5) flagellar preparation.

(x 30,000)

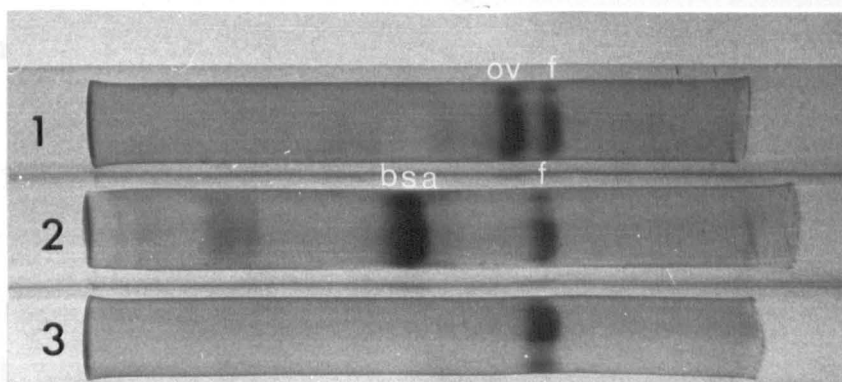


Plate 3.19

6% (w/v) polyacrylamide gels, 5mA/gel.

Standards. Bovine serum albumin, MW 68,000.

Ovalbumin, MW 46,000.

1. Ovalbumin, flagellin.
2. Bovine serum albumin, flagellin.
3. Flagellin.

to procaryotic resting cells (Sudo and Dworkin, 1973) and were first noted by Gorlenko (1969). These cells were formed in profusion by Rhodomicrobium (Rm5) and have been studied in considerable detail.

a) Spore formation.

Induction of sporulation usually involves a depletion of some growth supporting metabolite or accumulation of a catabolite (Halvorson, 1965; Murrell, 1967). The formation of Rhodomicrobium exospores conforms to this hypothesis in as much as they are observed only in the stationary phase of growth i.e. when nutrient exhaustion and catabolite accumulation occurs.

Slide cultures of Rhodomicrobium (Rm5) growing in a system of limited nutrient concentration revealed after 3-4 generations of vegetative growth, the mode of exospore formation. The angular cells are formed in succession from the filament tips by budding, only one cell being formed at a time (plate 3.20). Upon completion the exospore is physically released, although it may remain attached to the filament by the adherence of the capsular material (plate 3.21,3.23), the filament extends and synthesis of the second cell is initiated (plate 3.21,3.22; figure 3.19). As with vegetative cell growth each mother cell was found to be capable of only forming up to four exospores. These, however, in contrast to vegetative chain cells were always sequentially formed from the same filament tip. A similar situation exists for swarm cell production, i.e. a cell is formed and subsequently released, the filament then extends and synthesis of the second cell is initiated. The factor of four explains the high numbers of exospores encountered in the stationary phase, i.e. there is a potential of four exospores per mother cell. There is



Plate 3.20

Slide culture developmental sequence of exospore formation in Rhodomicrobium (Rm5). Phase contrast, (x 1250).

Gold palladium shadowed preparation of an isolated Rhodomicrobium (Rm5) resting cell showing irregularly and capsular stained material. (x 25,000)

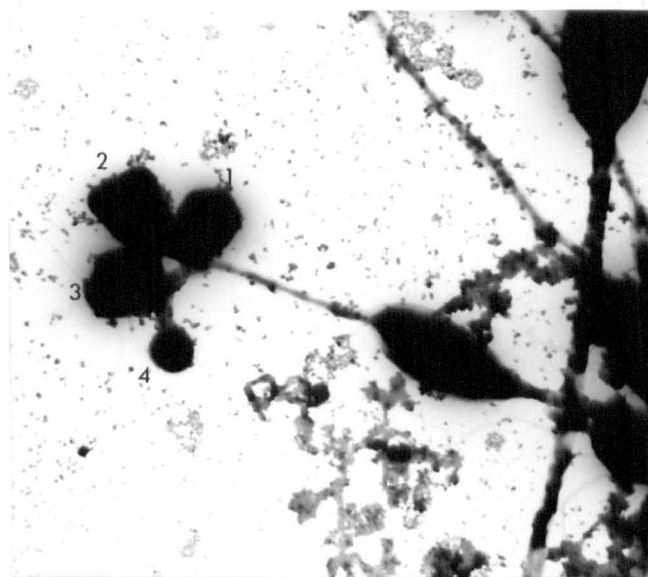
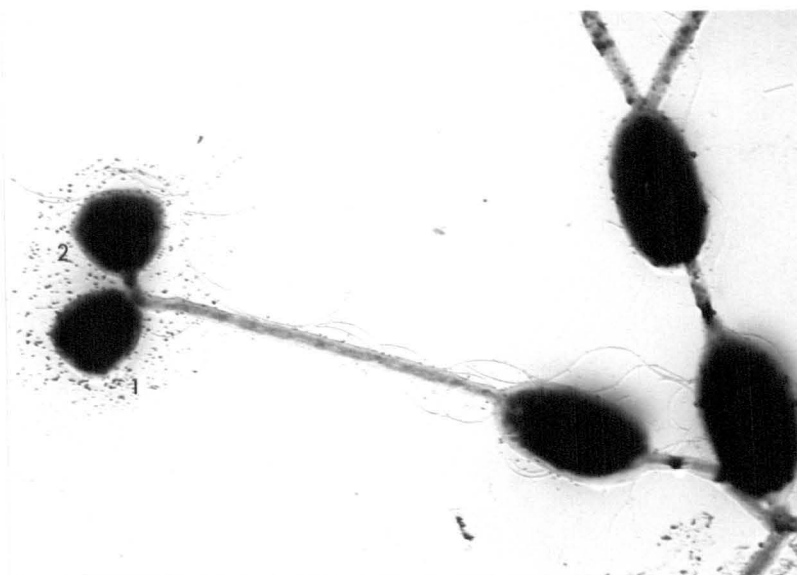


Plate 3.21 and 3.22

Gold palladium shadowed electron micrographs of exospore formation at the filament tip. (x 12,000)

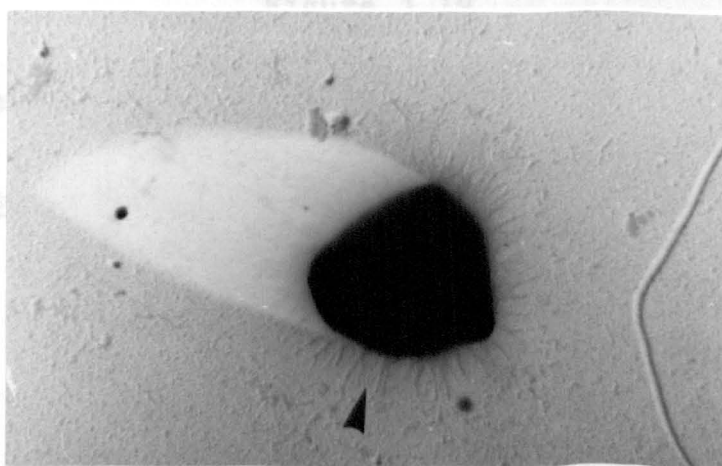


Plate 3.23

Gold palladium shadowed preparation of an isolated Rhodomicrobium (Rm5) resting cell showing angularity and capsular strand material. (x 25,000)

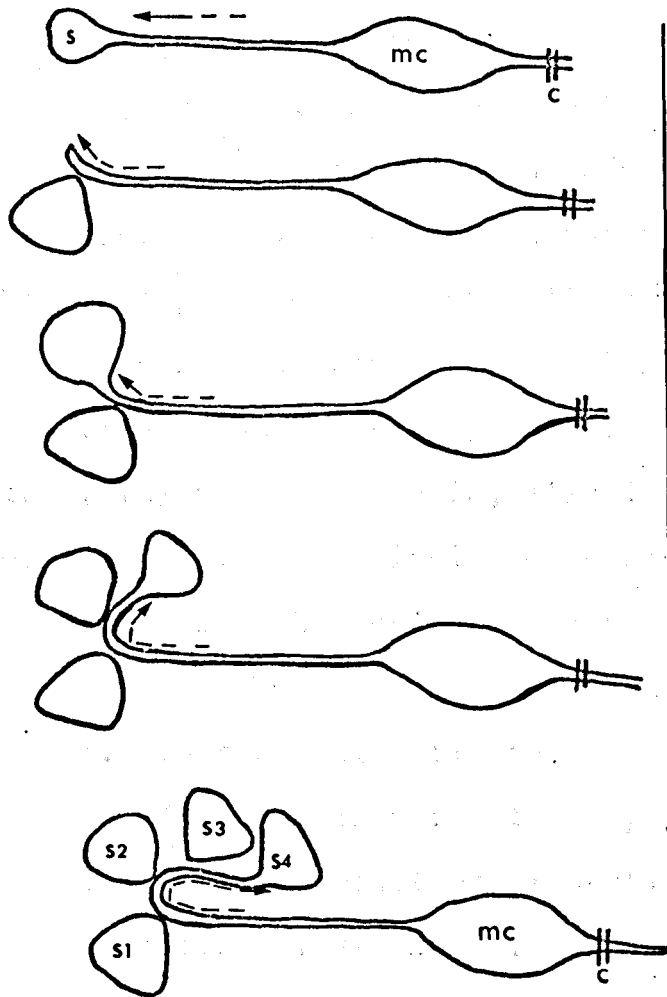


Figure 3.19

Schematic representation of spore formation in
Rhodomicrobium (Rm5).

not a one to one relationship between vegetative cell and spore as encountered with bacterial endospore formation.

b) Spore germination and outgrowth.

Activation, germination and outgrowth are the primary sequential processes known to occur in endospore to vegetative cell differentiation (Gould and Hurst, 1969). Although Rhodomicrobium exospores conform overall to this pattern, they are unique in several ways.

Inoculation of an exospore population into fresh liquid medium (PYR) under anaerobic, illuminated conditions, induces exospore germination within 3-4 hours. The sequence of events occurring in such a culture may be summarised as follows:

- | | |
|--------------------|--|
| | 1. Loss of refractility. |
| <u>Germination</u> | 2. Increase in permeation by specific dyes. |
| | 3. Loss of resistant properties. |
| | 4. Filament synthesis. |
| <u>'outgrowth'</u> | 5. Vegetative cell formation. |
| | 6. Sequential appearance of up to four daughter, vegetative cells. |

Germination

1. Loss of refractility

The decrease in optical extinction of visible light by a germinating exospore suspension was followed by phase contrast microscopy. The extent of this change in Rhodomicrobium exospores was however slight in comparison to that characteristic of Bacillus subtilis endospore germination (Hitchins et al, 1963). This may be explained in terms of the particular ultrastructural and physiological properties of the Rhodomicrobium exospore. Structurally it seems less complex than an endospore and the subsequent outgrowth processes are also

very different from those of the endospore. Endospores undergo 'complete' differentiation to a vegetative cell, whereas the exospore of Rhodomicrobium, upon germination, becomes a viable reproductive unit (as will be described later).

2. Increase in permeability.

Within 30 minutes of incubation in fresh medium, an exospore preparation stained with Malachite green/basic fuchsin and examined microscopically, shows a complete loss of resistance to permeation by dyes. This change is closely linked with the loss of refractility, indicating that both are associated with early physiological or ultra-structural alterations.

3. Loss of resistant properties.

A germinating exospore population sampled at intervals and assayed for heat resistance (90°C for 5 minutes) showed a marked and characteristic drop in heat resistance which was coincidental with loss of refractility and increased dye permeability.

These preliminary events are similar in sequence, although not extent, for all known procaryotic resting cells (spores and cysts). However, the subsequent developmental steps are unique and characteristic only of Rhodomicrobium.

4., 5., and 6. Outgrowth

Vegetative cell synthesis from the exospore is by the characteristic budding mode of growth being preceded by filament formation from one of the apices of this consistently angular cell (plate 3.24,3.26). The so formed daughter cell is morphologically indistinguishable from an exponential vegetative cell and consistent with this system is upon

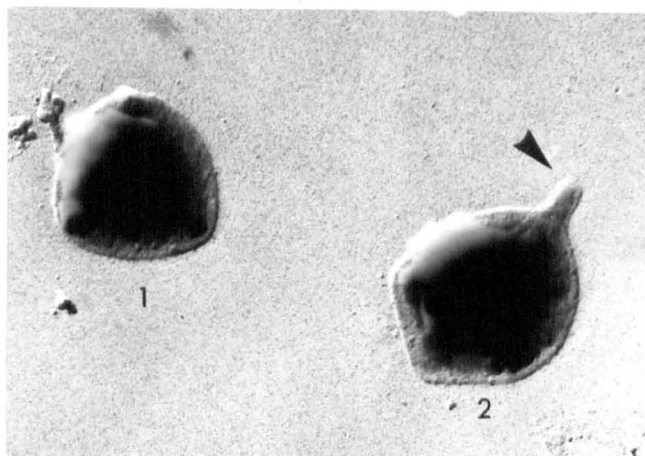


Plate 3.24

Rhodomicrobium (Rm5) resting cells; Dormant resting cell (1) and initiation of germination characterised by filament formation (2). Gold palladium shadow (x 20,000).

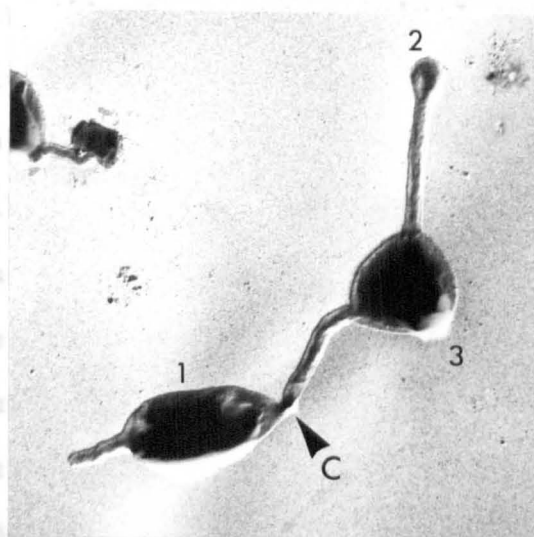


Plate 3.25

Germinating Rhodomicrobium (Rm5) resting cell showing first daughter cell (1), constriction formation (c) and initiation of synthesis of second daughter cell (2). (3) represents the site of formation of the third cell.

(x 12,000)

maturity isolated from the mother cell by a filament 'plug' (plate 3.25). The subsequent daughter cells are, however, formed by one of two modes of growth:

- i) branch formation from the original filament.
- ii) sequential filament synthesis from the apices of the angular exospore.

i) As illustrated in plate 3.26 and diagrammatically in figure 3.20(i), growth may be by branching without further initiation of filament synthesis from the other apices of the angular cell, i.e. synonymous with a vegetative cell 'locked' in a chain formation. (This sequence is identical to a series of photographs published by Duchow and Douglas (1949)(figure) as the vegetative cell cycle of Rm. vanniellii. Their method of slide culture preparation was conducive primarily to spore survival and in consequence, upon incubation, to germination.) The system is restricted, however, to observance of the same fundamental rules found to be applicable to vegetative cell growth. The distinctive feature is the capacity of the exospore to produce more than one vegetative cell, i.e. it has in effect become a replication unit synonymous with a normal vegetative cell.

ii) The sequential synthesis of filament and daughter cell formation from each apex of the 'triangular' exospore is unique to, and solely characteristic of the exospore. This mode of growth is illustrated in plate 3.27 and figure 3.20(ii). Not only is there differentiation of the exospore to a reproductive vegetative system with the potential to form up to four daughter cells, there is also a spatial arrangement of cell synthesis. Upon maturation of the first daughter

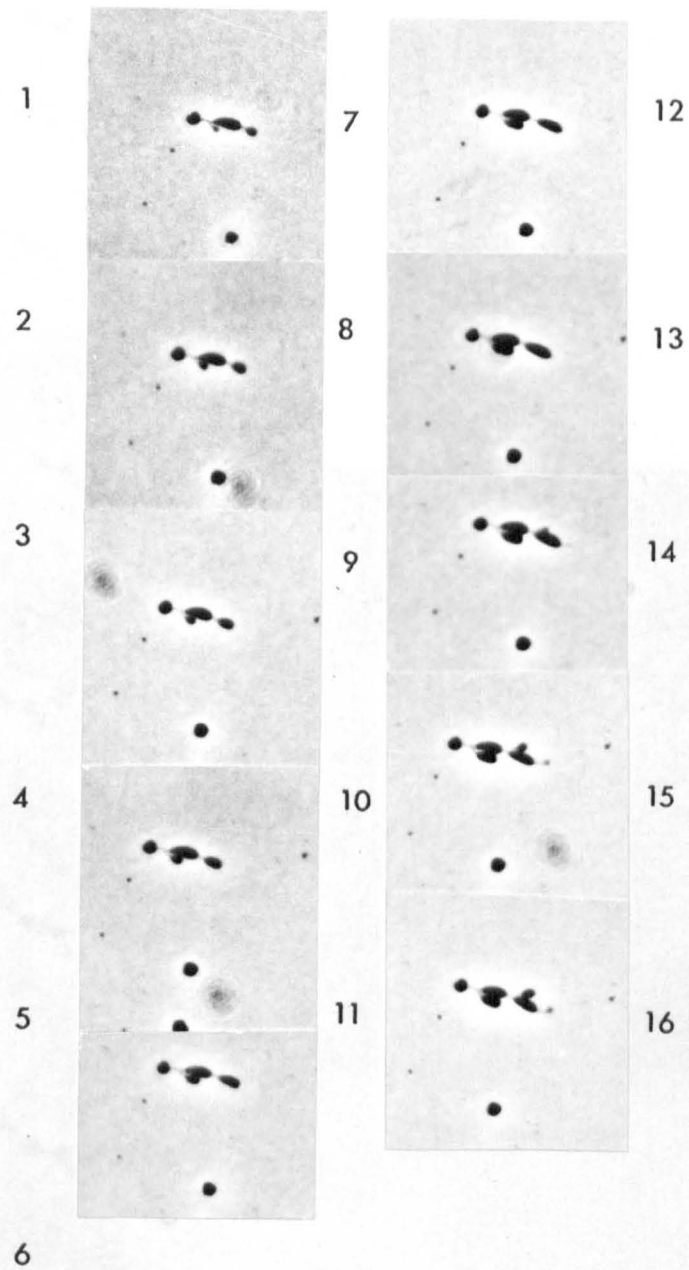
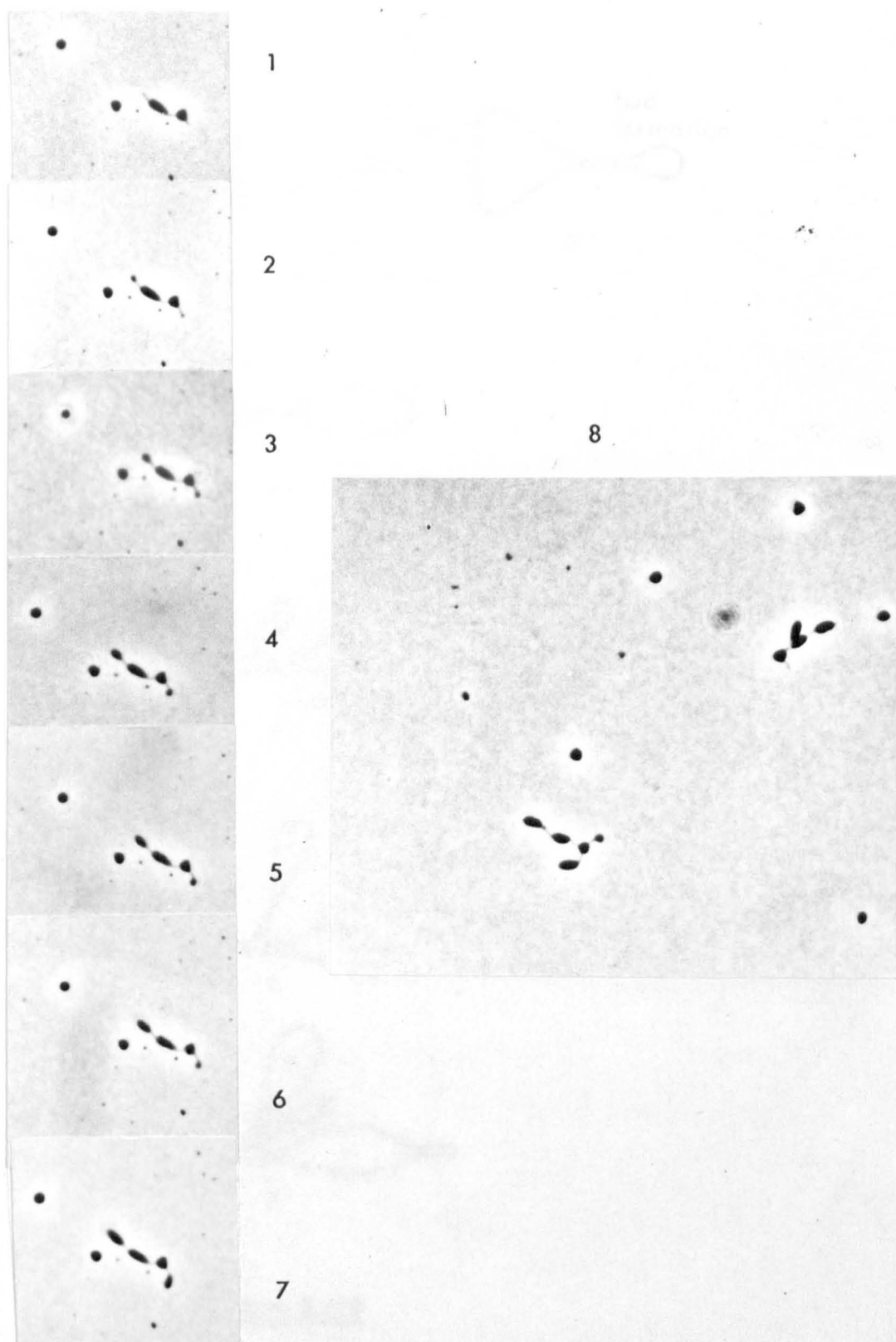


Plate 3.26

Vegetative cell formation from Rhodomicrobium (Rm5) exospores.

Phase contrast photomicrograph (x 1100)



(i) Exospore germination of *Rhodomicrobium* (Rm5) showing vegetative cell formation from exospores. Phase contrast photomicrograph (x 1100).

Plate 3.27

Vegetative cell formation from *Rhodomicrobium* (Rm5) exospores. Phase contrast photomicrograph (x 1100).

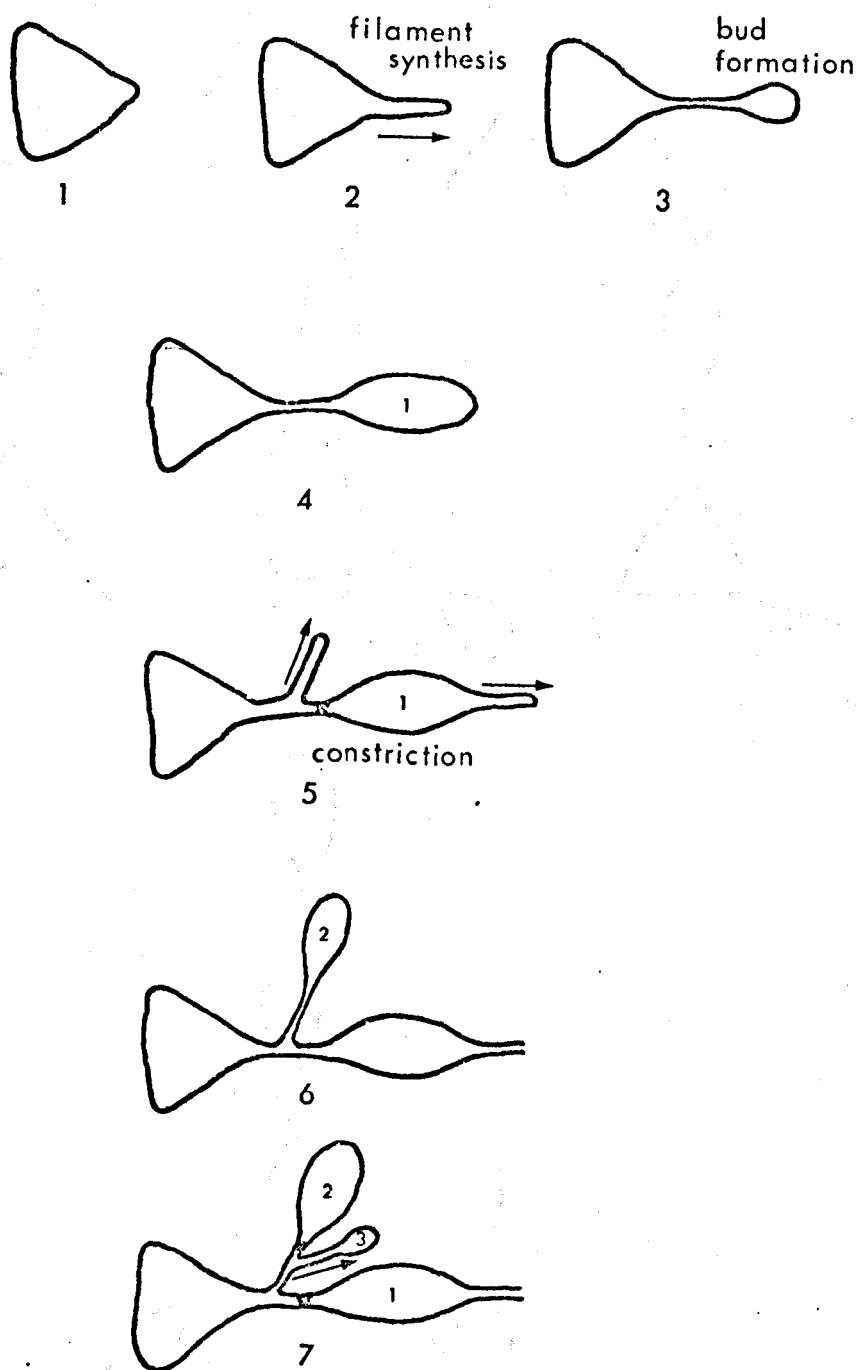


Figure 3.20

(i) Exospore germination with vegetative growth by filament branching.

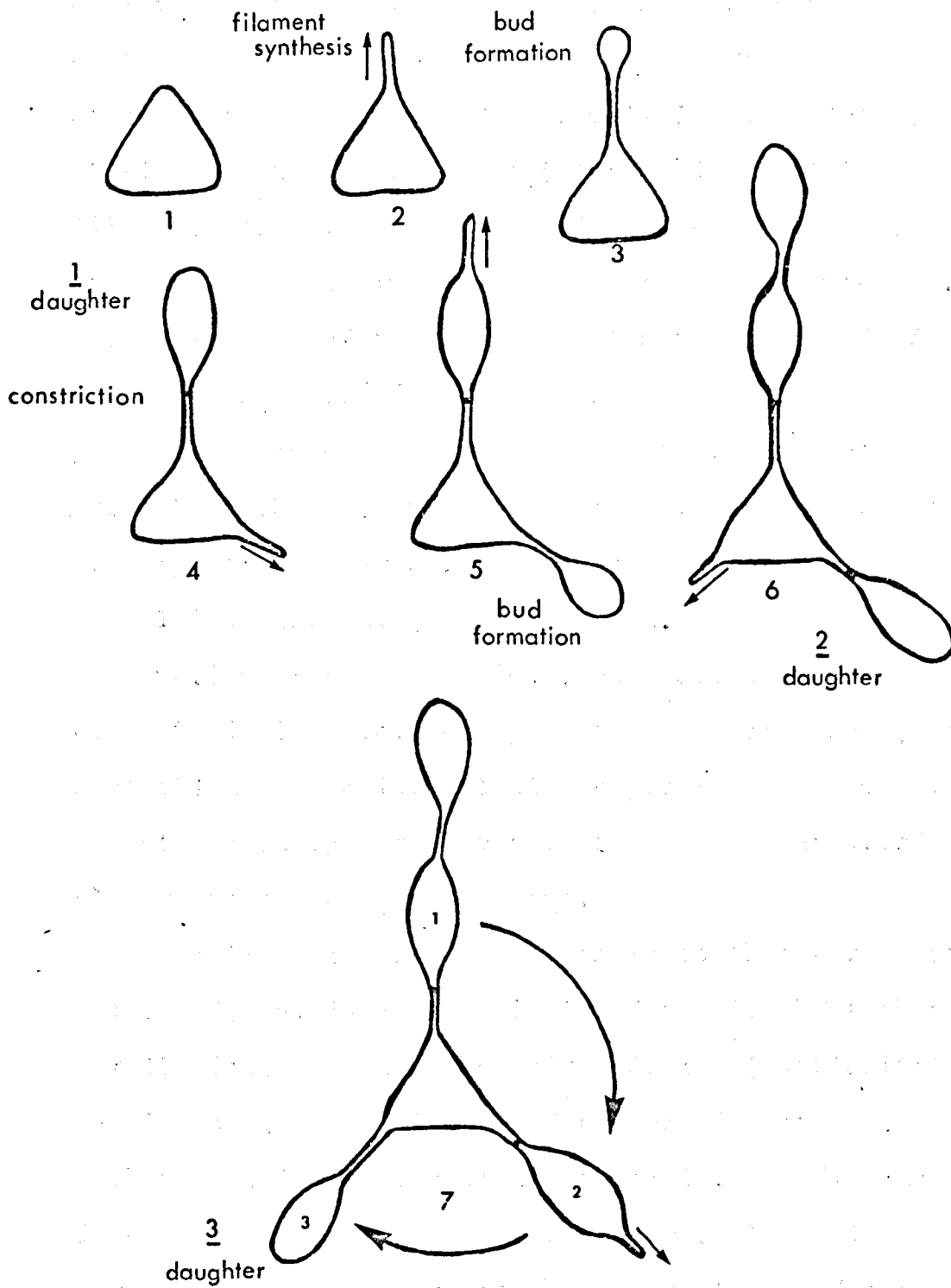


Figure 3.20

(ii) Exospore germination by sequential synthesis at each apex.

cell i.e. 'plug' formation, instead of initiating branch synthesis as in plate 3.26, a second filament is formed at another apex of the angular cell (plate 3.27(1)). Completion of this second cell (plate 3.27) is followed by filament formation at the third apex giving rise to the characteristic cellular arrangement shown in plate 3.27(8). The derivation of this unique cellular cluster is diagrammatically summarised in figure 3.20(ii).

Rhodomicrobium exospores are therefore intriguing not only with respect to their relationship to other known procaryotic resting cells but because of the physiological and ultrastructural questions posed. More importantly, germination provides a unique, model cellular system of differentiation i.e., exospore to vegetative cell, and of controlled sequential cellular expression physiologically and structurally strictly orientated and controlled.

c) Activation and germination.

Preliminary experiments indicate that germination is activated, initiated or stimulated by mild heat shock (55°C for 15 minutes) and amino acid addition (particularly alanine and lysine). In addition and of more importance, light, although it is not required for initiation of germination, is essential for subsequent daughter cell development. This was shown by following exospore germination under anaerobic light and dark conditions. Only under anaerobic light conditions was normal vegetative growth induced. Although germination was observed in aerobic dark conditions, daughter cell formation was abortive (plate 3.28). This developmental sequence suggests the use of an endogenous energy supply to initiate daughter cell formation but is then dependent on photosynthesis and concomitant metabolism for further

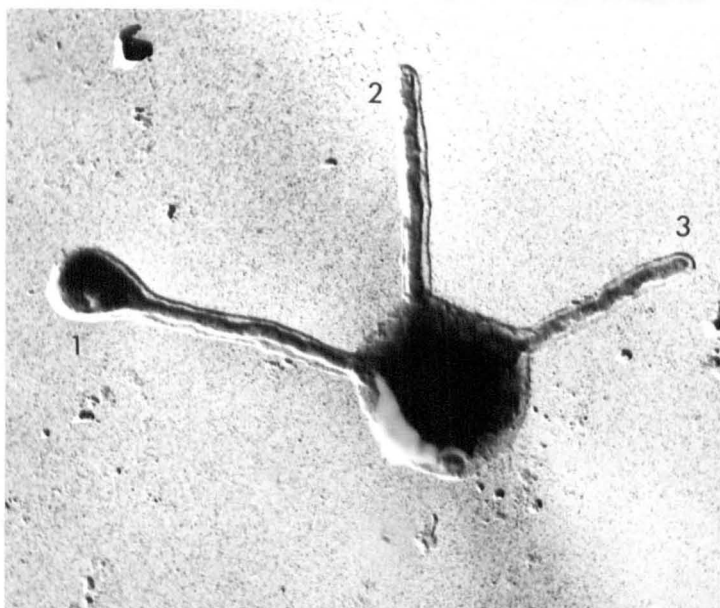


Plate 3.25

Phase contrast photomicrograph of Rhodocyclidium (Rm5) germinating exospore. (x 1150)

Plate 3.28

Abortive cell formation in Rhodocyclidium (Rm5) germinating exospore during growth under aerobic dark conditions. Gold palladium shadow (x20,000)

Plate 3.29

Interference photomicrograph of exospores. (x 1150)



Plate 3.31

Gold palladium shadowed electron micrograph of exospores

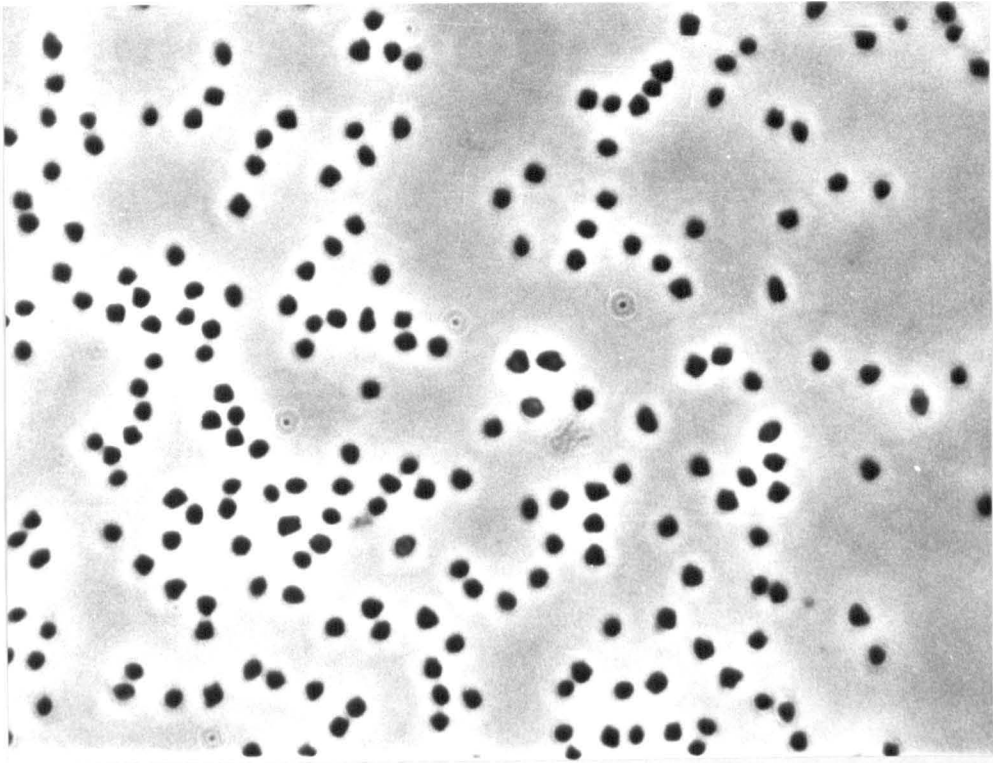


Plate 3.29

Phase contrast photomicrograph of Rhodimicrobium (Rm5) exospore preparation. (x 1250)

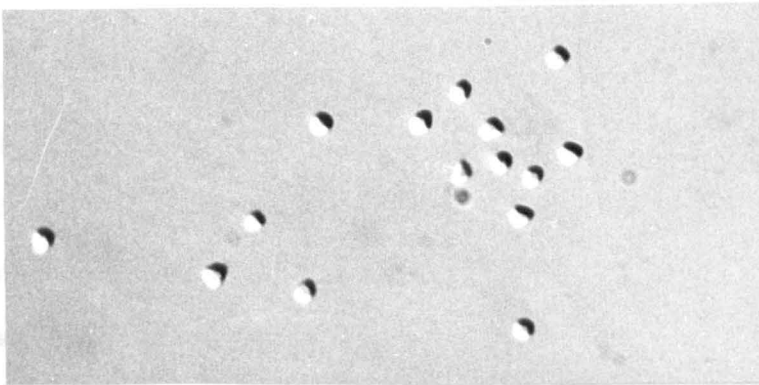


Plate 3.30

Interference photomicrograph of exospores. (x 1250)

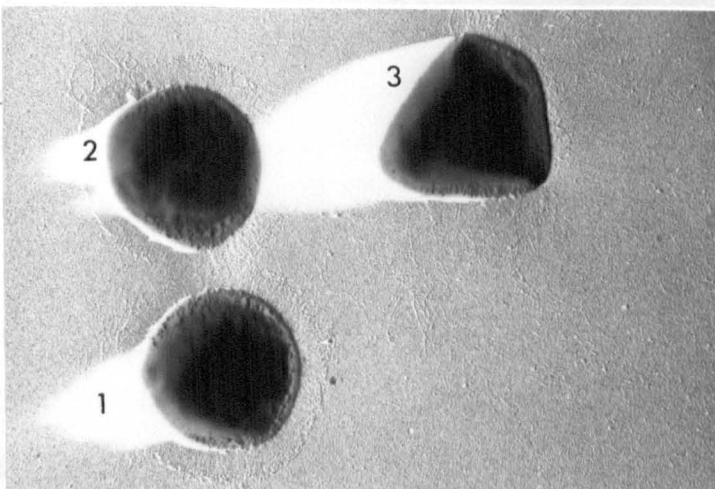


Plate 3.31

Gold palladium shadowed electron micrograph of exospores showing variation in angularity. (x 20,000)

growth. These observations suggest that the exospore is capable only of photosynthetic metabolism which would explain the thresh-hold levels of photosynthetic pigments found in the mature exospore (described below).

d) Exospore characteristics.

In size, the mature exospores vary considerably and although their shape is somewhat variable, a tendency toward angularity is always maintained. The overall cellular diameter is 0.25-1.5 μ (plate 3.29,3.30,3.31). The filaments and daughter cells formed by germinating spores are morphologically indistinguishable from those formed by normal vegetative cells.

1. Stainability.

Exospore suspensions obtained by centrifugation and filtration (section 3.2(r)) when subjected to staining with Malachite green (Ashby, 1938) and several simple dyes revealed that only 75% of the population was resistant to permeation i.e. indicative that a significant proportion of the population was immature or alternatively, had initiated germination. As with most types of spores (Gould and Hurst, 1969), resistance to dye permeation is lost relatively early in the germination sequence.

2. Heat resistance.

Heat treatments performed on exospore suspensions demonstrated considerable tolerance to high temperatures (table 3.5). Detailed analysis of tolerance to three temperatures, 50⁰, 60⁰ and 80⁰C, consistently produced non-linear plots (figure 3.21) despite extreme precautions to ensure heat equilibration of the test suspension. These results are typical of inactivation of a mixed cellular suspension,

Table 3.5

Heat tolerance (all or none) by Rhodomicrobium (Rm5)
exospores, vegetative cells and Rhodopseudomonas palustris.

Temp.(°C)	Time (mins.)	Exospores	Growth veg.cells.	<u>Rh.</u> <u>palustris</u>
40	30	+	+	+
50	20	+	-	-
60	20	+	-	-
70	20	+	-	-
80	15	+	-	-
90	5	+	-	-
100	5	(+)	-	-
100	10	-	-	-

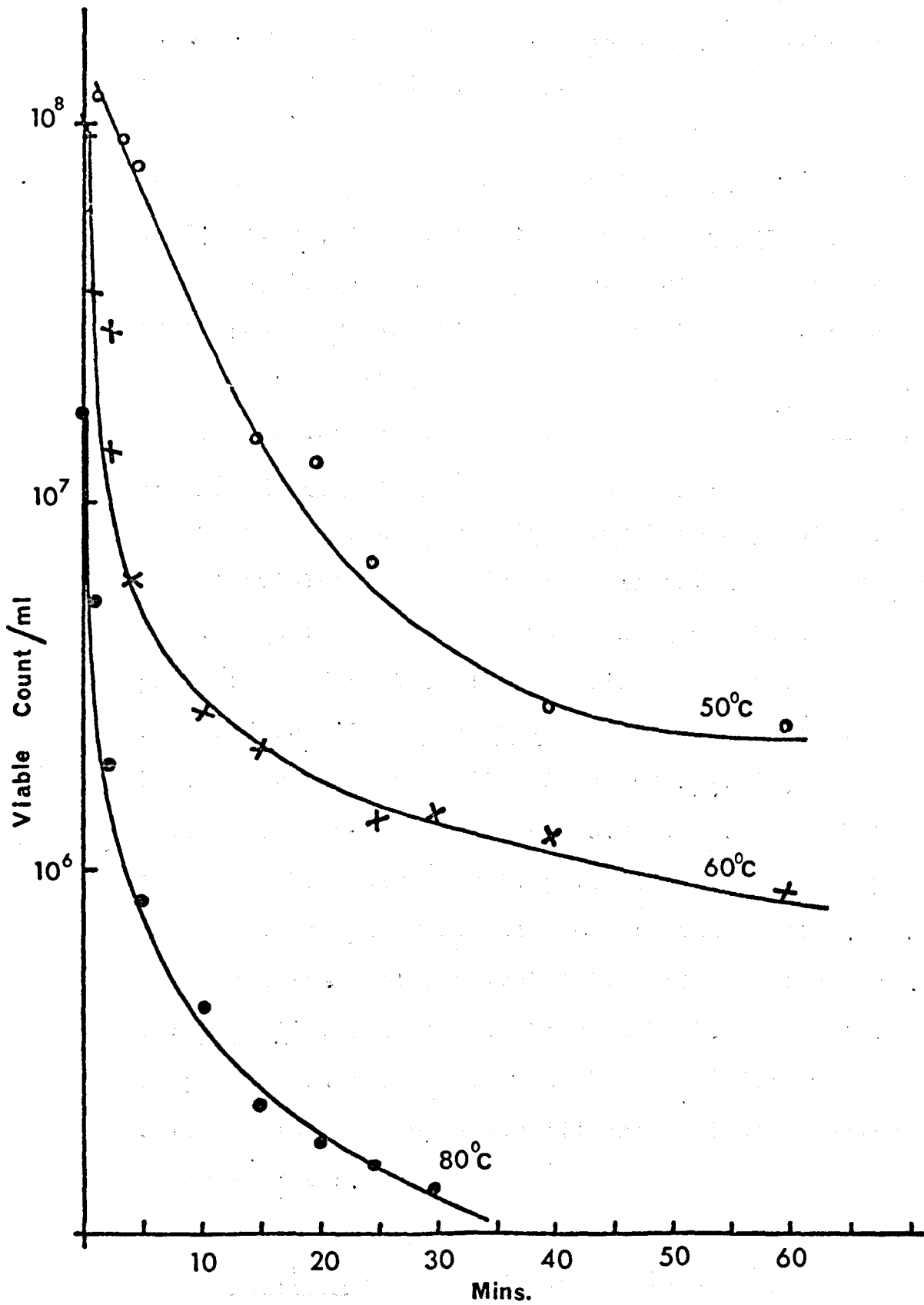


Figure 3.21

Survival curves of *Rhodomicrobium* (Rm5) exospores subjected to 50°, 60° and 80°C.

i.e. mature (dormant) and immature (germinating) spores. (Roberts and Hitchins, 1969).

In comparison to the heat resistant properties of the endospores of Bacillus or Clostridium (Roberts and Ingram, 1965; Briggs, 1966), the cysts of Azotobacter (Socolofsky and Wyss, 1962) and the myxospores of Myxococcus ruber (Bauer, 1905) the Rhodomicrobium exospores show a tolerance to heat approximate to that of myxospores (Sudo and Dworkin, 1973) and to the less resistant of the endospores (Briggs, 1966).

3. Ultraviolet irradiation resistance.

Exospores of Rhodomicrobium have a greater resistance to ultraviolet irradiation than is shown by the vegetative cell, requiring about 30% more energy to kill the same proportion of cells. In comparison 500% more energy is required to kill M. xanthus myxospores than is required to kill the vegetative cells (Sudo and Dworkin, 1973). Survival curves are non-linear (figure 3.22), further substantiating the idea that only a small percentage of the exospore suspension is composed of fully mature resistant cells. Plots of vegetative cell survival follow closely that determined for E. coli (although slightly higher). The mature exospore suspension on the other hand shows an initial sharp drop in survival followed by a significantly slower killing rate on further exposure.

4. Lysozyme resistance.

Bacillus endospores are not killed by lysozyme (Tonisik and Baumann-Grace, 1959). However, both vegetative cells and exospores of Rhodomicrobium are killed by exposure to high concentrations (4 mg/ml) at 30°C for 1 hr.

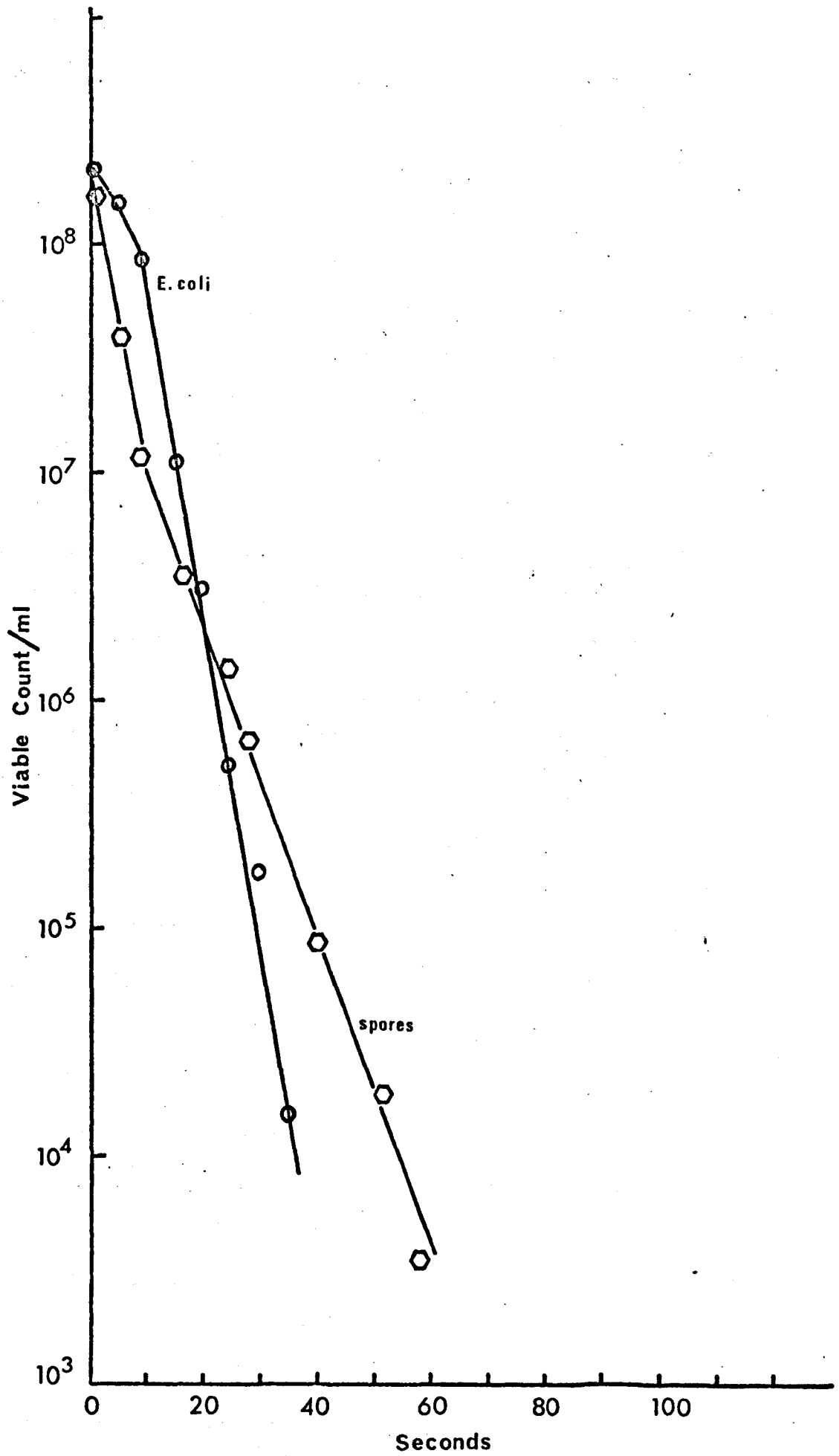


Figure 3.22
 UV survival curve of Rhodomicrobium (Rm5) exospores
 in comparison to that of an E. coli standard.

5. Dipicolinic acid content.

Both calcium and dipicolinic acid (DPA) have had ascribed to them a variety of functions in bacterial endospore resistance (Murrell, 1969). The apparent close association of DPA and calcium content with heat resistance during endospore formation and germination has lead to these materials being thought of as the prime factors involved in the heat tolerance of endospores (Halvorson and Church, 1957). However, Vinter (1962) found that DPA accumulation occurred far in advance of aquisition of heat resistance during sporulation of B. cereus. In addition, Zytkevicz and Halvorson (1972) isolated DPA-less mutants of B. subtilis which were initially heat resistant but lost this property with ageing. Murrell (1969) has compiled data which indicate that Ca^{2+} is probably more directly involved in heat resistance and dormancy than DPA. However, in view of the transient heat resistance of DPA-less mutants it seems likely that DPA is necessary to trap large amounts of divalent cations in a small volume, hence the close relationship between Ca^{2+} , DPA and heat resistance.

DPA is not, however, ubiquitously present in heat resistant cells being found only in procaryotic endospores (Sudo and Dworkin, 1973).

Rhodomicrobium (Rm5) exospores analysed by the procedure of Jansen et al. (1958) contain no DPA.

6. Spectral characteristics.

Figure 3.23 illustrates the spectral characteristics of an exospore suspension in comparison to that of an exponential, photosynthetically grown vegetative cell suspension having a similar optical density at 540 nm. To ensure there

was no contamination of the exospore suspension by photopigments of vegetative cell origin the following purification procedures were employed sequentially:

- i) centrifugation
- ii) column filtration
- iii) sucrose gradient sedimentation

No ovoid i.e. vegetative cells, were present in an exospore suspension prepared in this manner (as determined microscopically).

There is a notable and quantitatively significant reduction in both chlorophyll and carotenoid content of the exospores. Qualitatively however, the photopigments of both cell types are identical.

The 'residual' photopigment content of the exospore may be associated with the observed disorientation of exospore outgrowth when germinated aerobically in the dark. That is to say, although initial germination sequences up to filament formation appear to be dependent solely on endogenous energy (since both aerobic/dark and anaerobic/light germinated exospores are similar up to this point) further development and synthesis of the daughter cell is dependent on energy derived from activity of the inherent "threshold" photosynthetic pigments, i.e. light is required. The difficulty with this interpretation is that one is effectively saying that the germinating exospore is incapable of deriving energy by aerobic/dark chemoorganotrophic metabolism, a property not, however, characteristic of the vegetative cell. An alternative explanation is that the photopigment content is a manifestation of the 'immature' proportion of the population demonstrable from cell resistance studies. Both

possibilities have evidence for and against them. Clarification of the derivation and physiological implications of this 'residual' photopigment content (if it is such) requires more detailed investigation.

E) Ultrastructure of *Rhodomicrobium* cell types.

Several ultrastructural studies of *Rhodomicrobium* have been documented. The original work by Vatter et al, (1959) demonstrated a lamellar membrane system which was subsequently verified by Conti and Hirsch (1965) in comparative studies with *Hyphomicrobium*. The most recent work by Trentini and Starr (1967) not only illustrates the characteristic membrane system but associates the extent of lamellar membrane network with light intensity. None of these workers were conversant, however, with the cellular types and development characteristics of this organism and in consequence gave no indication as to the ultrastructural organisation necessary for cellular replication.

(i) Vegetative cell(chain formation).

Plate 3.32 is an electron micrograph of a transverse section of a *Rhodomicrobium* cell from an exponential culture grown at a light intensity of 800 lux (Acrolein, glutaraldehyde, potassium permanganate fixation). The presence of poly- β -hydroxybutyrate (PHB) and the double filament extension are indicative that this is a mature cell 'locked' within a chain sequence. The lamellar membrane system and the discrete cytoplasmic membrane are clearly seen. Additionally the continuity of the cell cytoplasmic membrane and wall with the filament is obvious. The cell wall structure is characteristic of, and identical to that of other Gram -ve bacteria (Forsberg, Costerton and MacLeod, 1970).

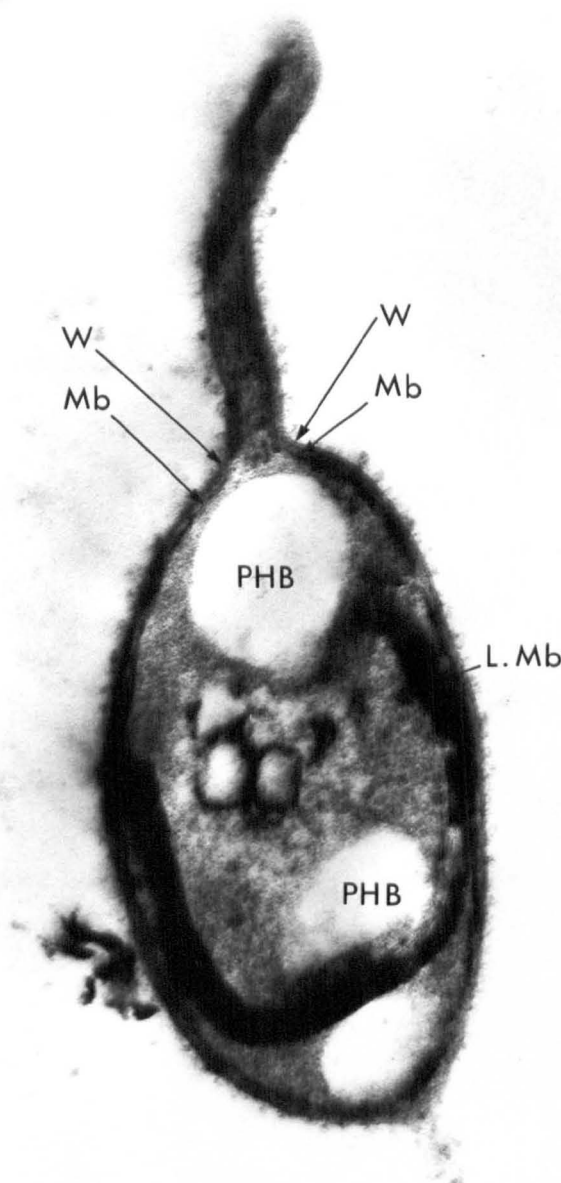


Plate 3.32

Transverse section of a vegetative cell of Rhodomicrobium (Rm5) showing continuity of cell wall (w) and cytoplasmic membrane (Mb) with those of the filament, characteristic lamellar membrane system (L.Mb.) and poly hydroxybutyrate inclusion granules (PHB).

(Alcolein, glutaraldehyde, potassium permanganate fixation)

(x 60,000)

(ii) Swarm cell.

The peripheral lamellar membrane system, the cytoplasmic membrane and cell wall of the swarm cell are microscopically indistinguishable from those of chain-forming vegetative cells. Plates 3.33 and 3.34 which are transverse and cross sections of swarm cells, illustrate this.

(iii) Exospore ultrastructure.

Cross and transverse sections of exospores reveal, as far as can be ascertained, that the membrane and cell wall are indistinguishable from those associated with swarm and vegetative cells, i.e. there are no apparent exospore specific layers (plate 3.35). However, although the membrane system is complex and prolific, from spectral absorption studies (figure 3.23) there is a significant discrepancy between membrane and photosynthetic pigment content. The resistant properties may therefore reside in a differential membrane system as opposed to cell wall modification or addition of specific layers. Alternatively there exists the possibility that a 'cortical' layer is formed by intercallation of material with the membrane system. However before one can ascertain the detailed ultrastructure of the dormant exospore it will be necessary to do sequential studies on the ultrastructure of exospore germination so correlating structural modifications (if any) with the loss of the characteristic resistance properties.

No procaryotic resting cells so far described bear ultrastructural similarity with the Rhodomicrobium exospores.

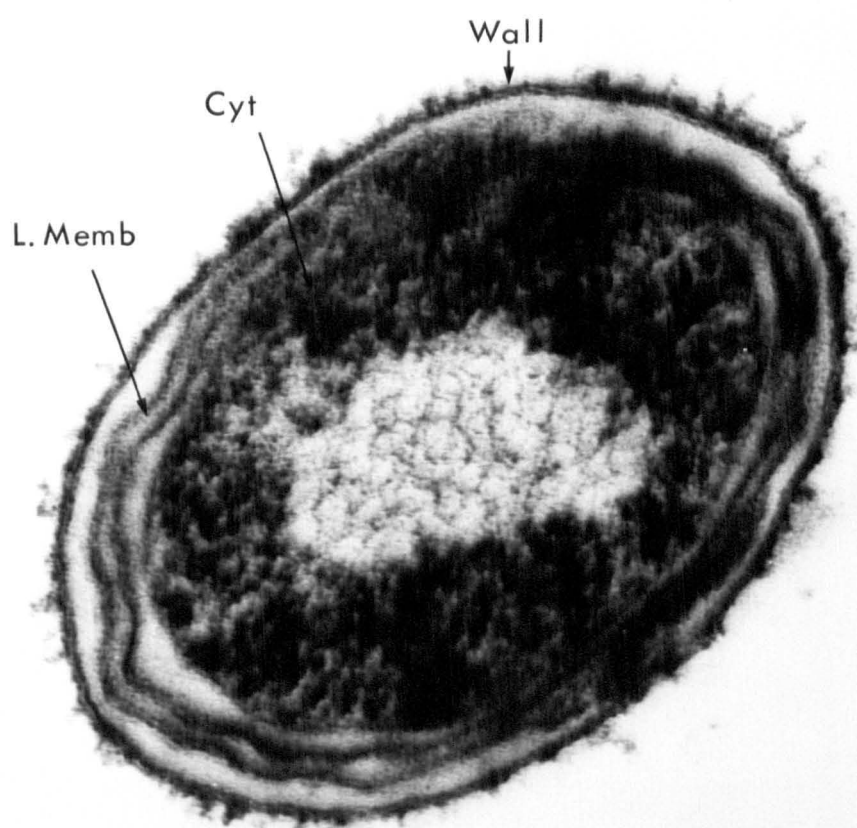
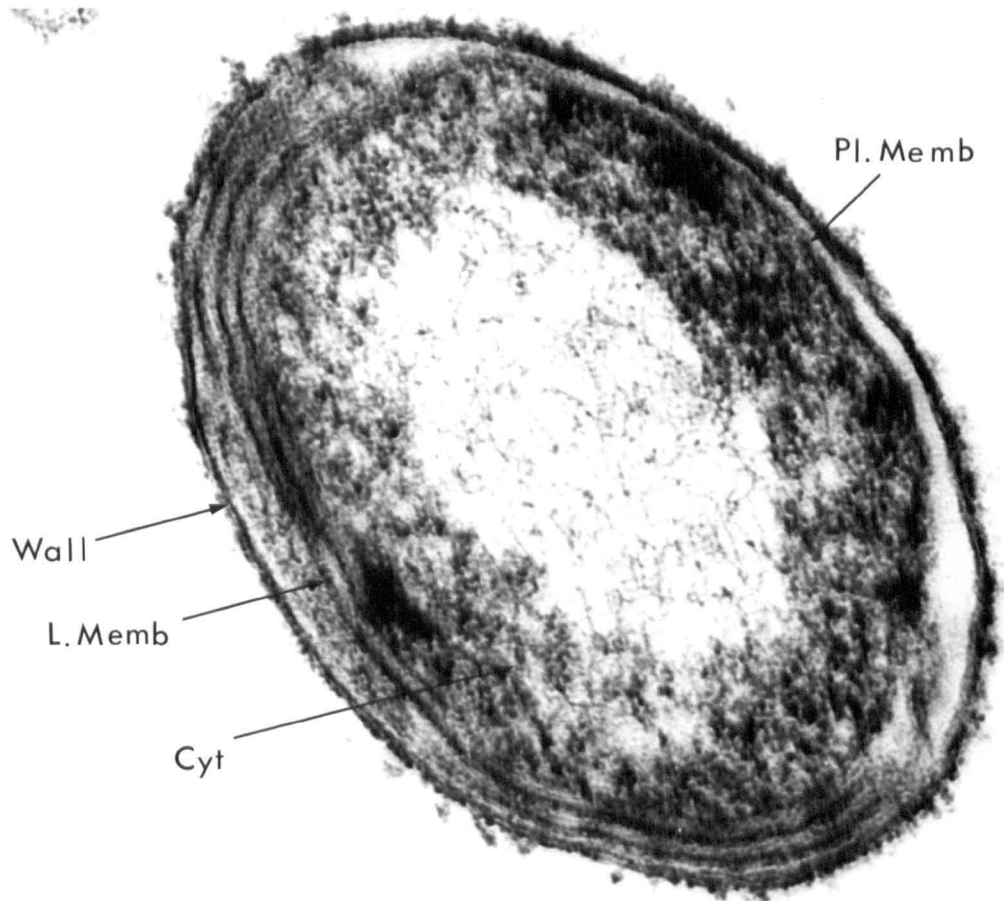


Plate 3.33, 3.34

Transverse (3.33) and cross-section (3.34) electron micrographs of Rhodomicrobium(Rm5) swarm cells. L.Memb.-Lamellar membrane, Pl.Memb.-Plasma membrane, Cyt.-cytoplasm. (Kellenberger fixation x 120,000)

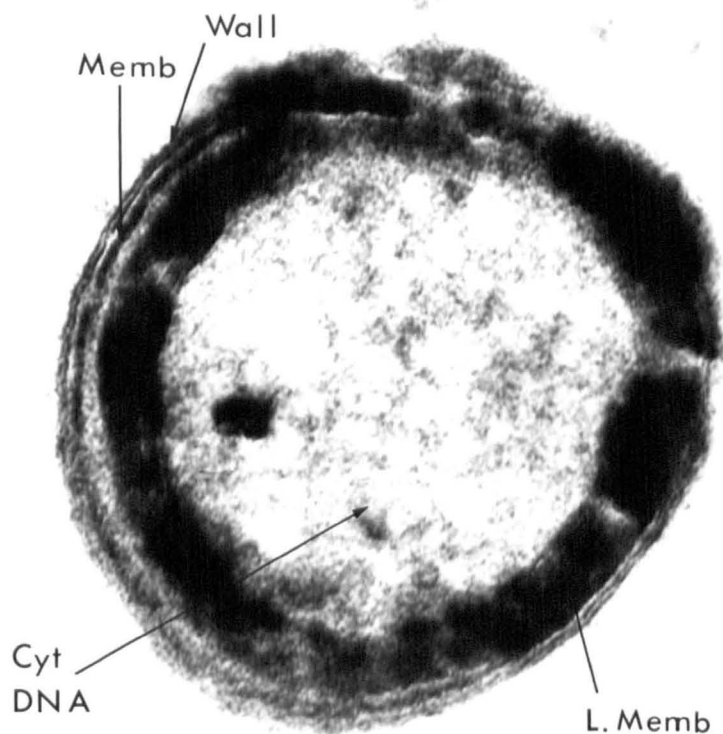


Plate 3.35

Transverse section of Rhodomicrobium (Rm5) exospore showing the complex lamellar membrane system (L.Memb.) and the cytoplasmic membrane (Memb.) (Acrolein, Glutaraldehyde, potassium permanganate fixation) (x 100,000).

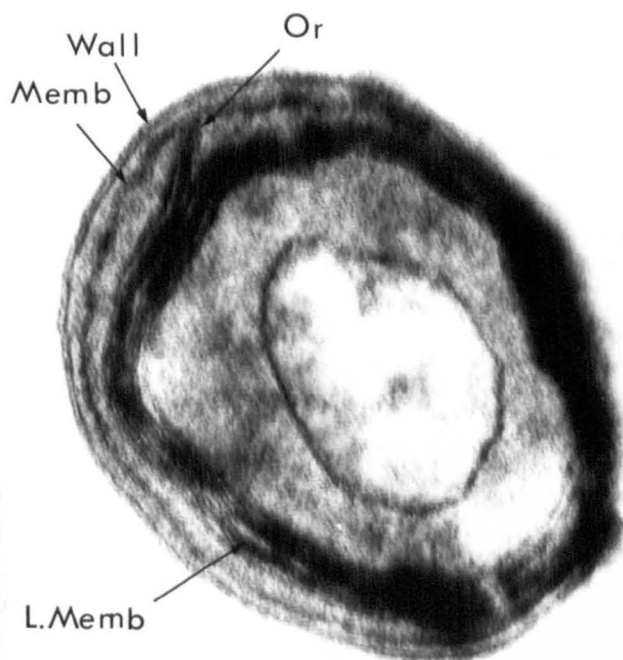


Plate 3.36

Cross section of Rhodomicrobium (Rm5) exospore showing the origin (Or) of the lamellar membrane system. (x 100,000)

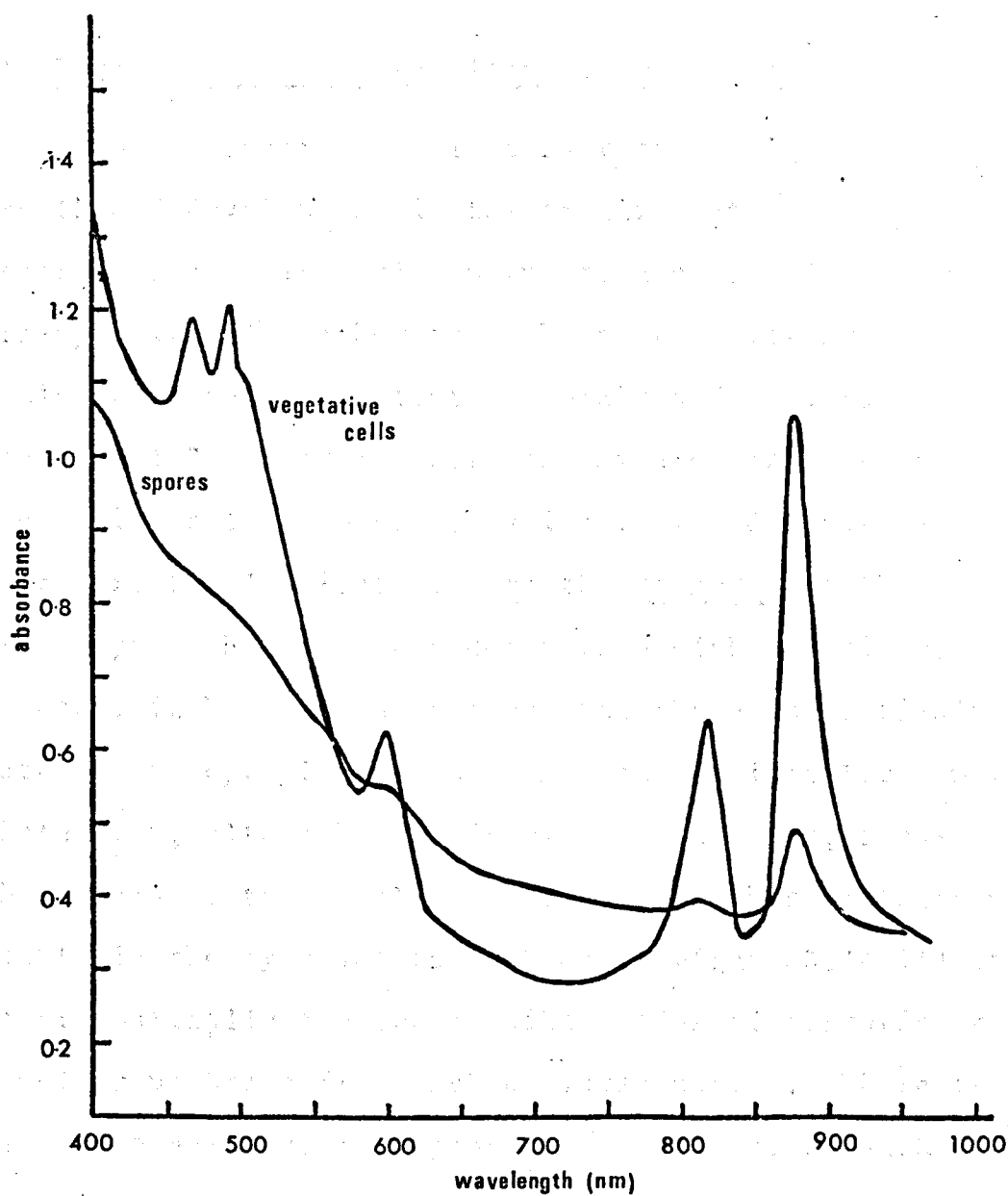


Figure 3.23

Absorption spectra of whole vegetative cells and exospores of Rhodomicrobium in saturated sucrose.

F) Derivation of the lamellar membrane system.

An important and unique characteristic of Rhodomicrobium growth and development is the requirement for de novo membrane synthesis during maturation of the daughter cells, irrespective of whether these be swarm cells or cells in a chain sequence. Radioisotope uptake and spectral studies (section 4.3) of swarm cells and mature first generation vegetative cells support this idea. The derivation and ultrastructural orientation of the de novo lamellar membrane system has not been determined with certainty. However, it has been suggested that there is continuity between the cytoplasmic membrane and the lamellar membrane. Plate 3.36, although of a developing exospore, shows that the origin of the lamellar system is in this instance undoubtedly the cytoplasmic membrane. Figure 3.24 illustrates diagrammatically the two possible modes of formation of the lamellar membrane from such an invagination. There is, however as yet no evidence to suggest the existence of such an invagination being the origin of the lamellar system in vegetative cells. What ultrastructural evidence pertaining to lamellar membrane synthesis is therefore available, and from this what are the possible ultrastructural models?

Models which explain the known ultrastructural membrane orientations may be formulated as follows. The origin of the cytoplasmic membrane may be in one of two planes, horizontal or vertical or possibly circular in nature. A horizontal invagination (figure 3.25,H) will give transverse and cross-sections as diagrammed in figure 3.25(A and B) but it becomes difficult to conceive a method of formation

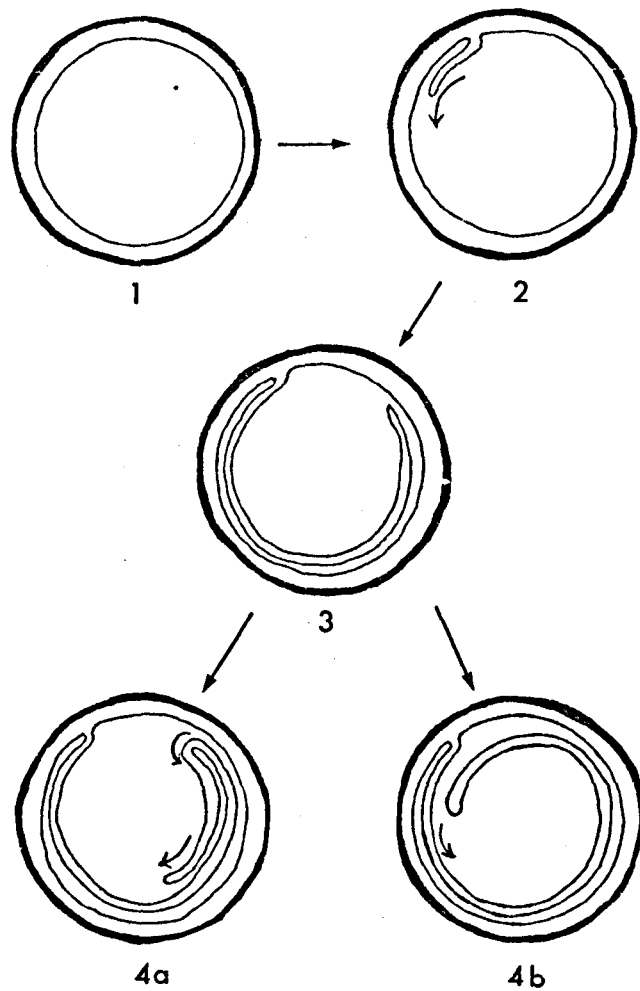


Figure 3.24

Schematic representation of lamellar membrane synthesis in the developing spore.

1. Newly formed spore.
2. Invagination of cytoplasmic membrane.
3. Growth of invagination.
- 4a. alternative modes of synthesis.
- 4b.

Figure 3.25

Diagrammatic representation of the possible derivation of the lamellar membrane system in Rhodomicrobium sps.

A and C: transverse sections.

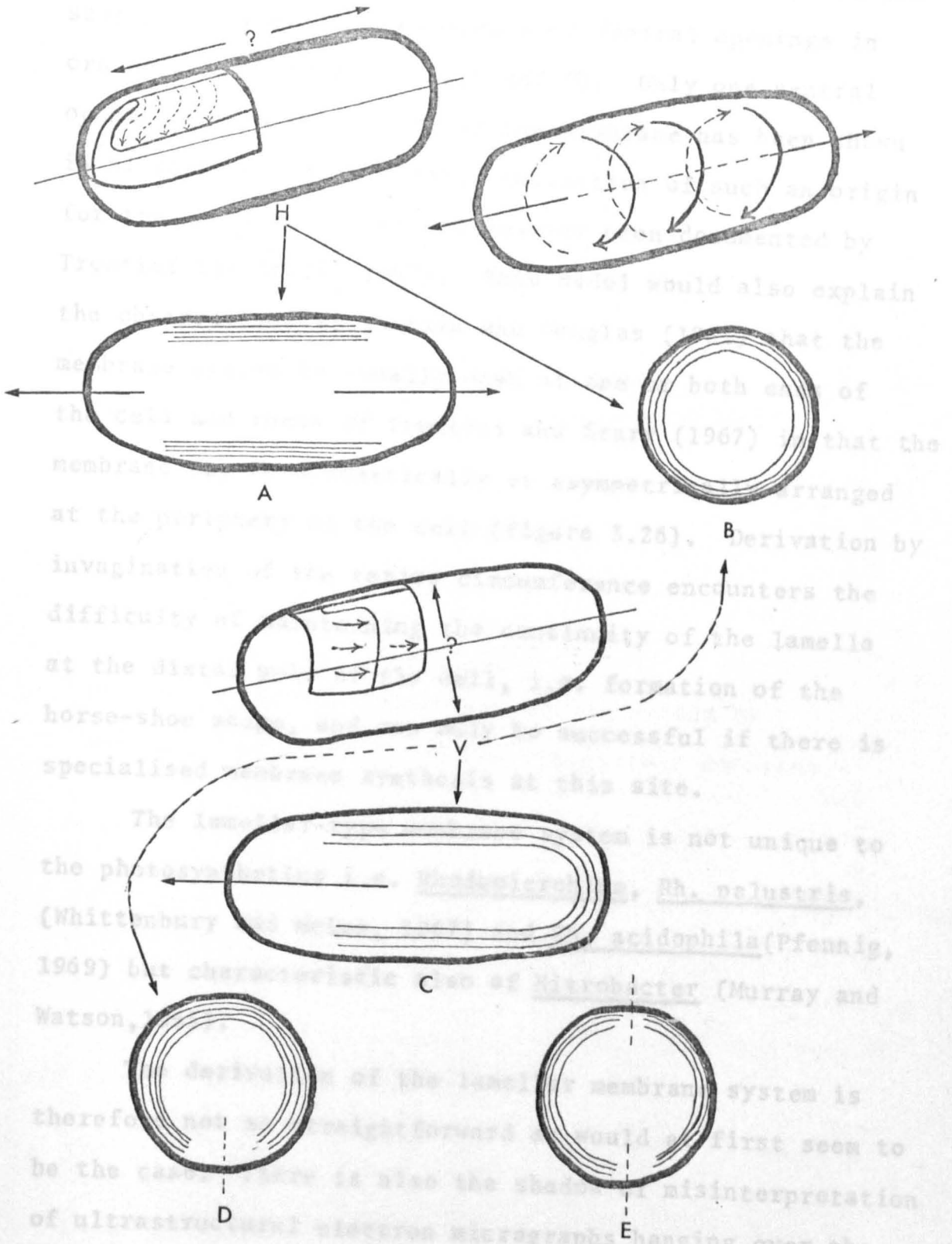
references: A. Trentini and Starr (1967)
C. Plate 3.32, this thesis.

B, D and E: cross sections.

references: B. Conti and Hirsch (1965)
D. Whittenbury and McLee (1967)
E. not recorded.

H and V: 3 dimensional orientation of membrane invagination.

references: H. plate 3.36, this thesis.
V. Trentini and Starr (1967).



of the characteristic horse-shoe orientation (figure 3.25, C). Cytoplasmic membrane invagination on a vertical plane (figure 3.25, V) would surmount this inconsistency but present yet another, the existence of ventral openings in cross-section (figure 3.25, D and E). Only one ventral opening (D) or continuity of the membrane has been shown in ultrastructural studies. Indication of such an origin for the lamellar system has however been documented by Trentini and Starr (1967). This model would also explain the observations of Boatman and Douglas (1961) that the membrane system is usually open at one or both ends of the cell and those of Trentini and Starr (1967) in that the membrane may be symmetrically or asymmetrically arranged at the periphery of the cell (figure 3.26). Derivation by invagination of the entire circumference encounters the difficulty of maintaining the continuity of the lamella at the distal pole of the cell, i.e. formation of the horse-shoe shape, and can only be successful if there is specialised membrane synthesis at this site.

The lamellar-type membrane system is not unique to the photosynthetics i.e. Rhodomicrobium, Rh. palustris, (Whittenbury and McLee, 1967) and Rh. acidophila (Pfennig, 1969) but characteristic also of Nitrobacter (Murray and Watson, 1965).

The derivation of the lamellar membrane system is therefore not so straightforward as would at first seem to be the case. There is also the shadow of misinterpretation of ultrastructural electron micrographs hanging over the work. However, ultrastructural studies on a sequence of samples of synchronised Rhodomicrobium swarm cells (section 4.3) and the germination sequences associated with the

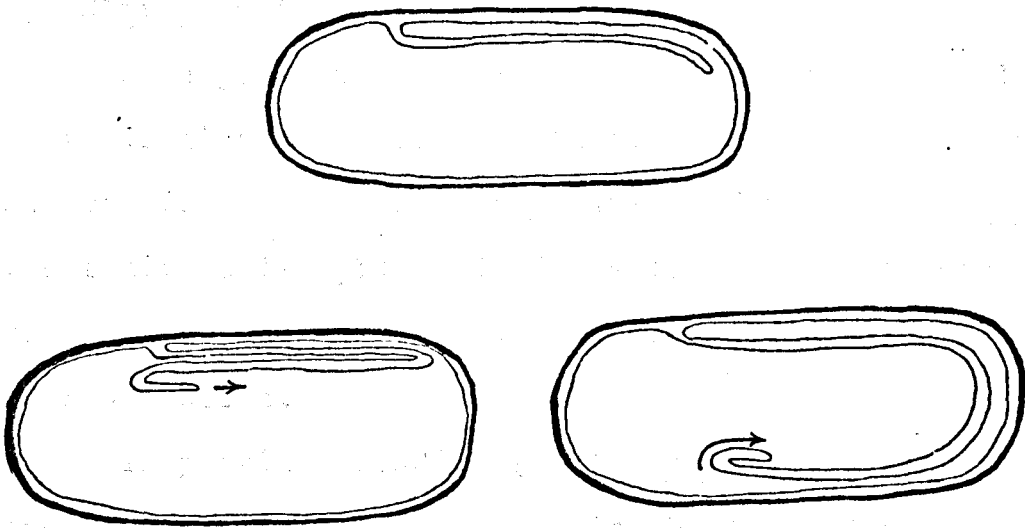


Figure 3.26

Derivation of the lamellar membrane system by
vertical invagination of the cytoplasmic membrane.

exospores should provide the necessary ultrastructural information for formulation and clarification of lamellar-membrane synthesis.

G) Ultrastructure of replication.

When grown under high light intensity (8,000 lux) there is little membrane synthesis but characteristic and consistent compartmentalisation of the Rhodomicrobium vegetative cell (plates 3.37-3.40). The interpretation assigned to this observation is correlated with the mode of replication.

In previous studies (Vatter et al, 1959; Boatman and Douglas, 1961; Conti and Hirsch, 1965; Trentini and Starr, 1967) the comparatively low light intensities (100 lux) required the cell to synthesise complex lamellar membrane systems which would mask most other ultrastructural features. Additionally the slow growth rates did not enhance or require the subdivision of the cell, a factor which will become apparent as this argument develops. Trentini and Starr (1967) did, however, in one instance comment on such compartmentalisation.

The complex but organised mode of growth of Rhodomicrobium has not only the problem of transport of materials to overcome, i.e. from mother cell to developing daughter, but also the added complication of a complex membrane system to by-pass during reproduction.

Detailed analysis of the ultrastructural electron micrographs of developing cells (plates 3.39-3.40) has allowed formulation of the following model. The compartmentalization of the mother cell can be explained as cellular replication and division within the confines of a fixed

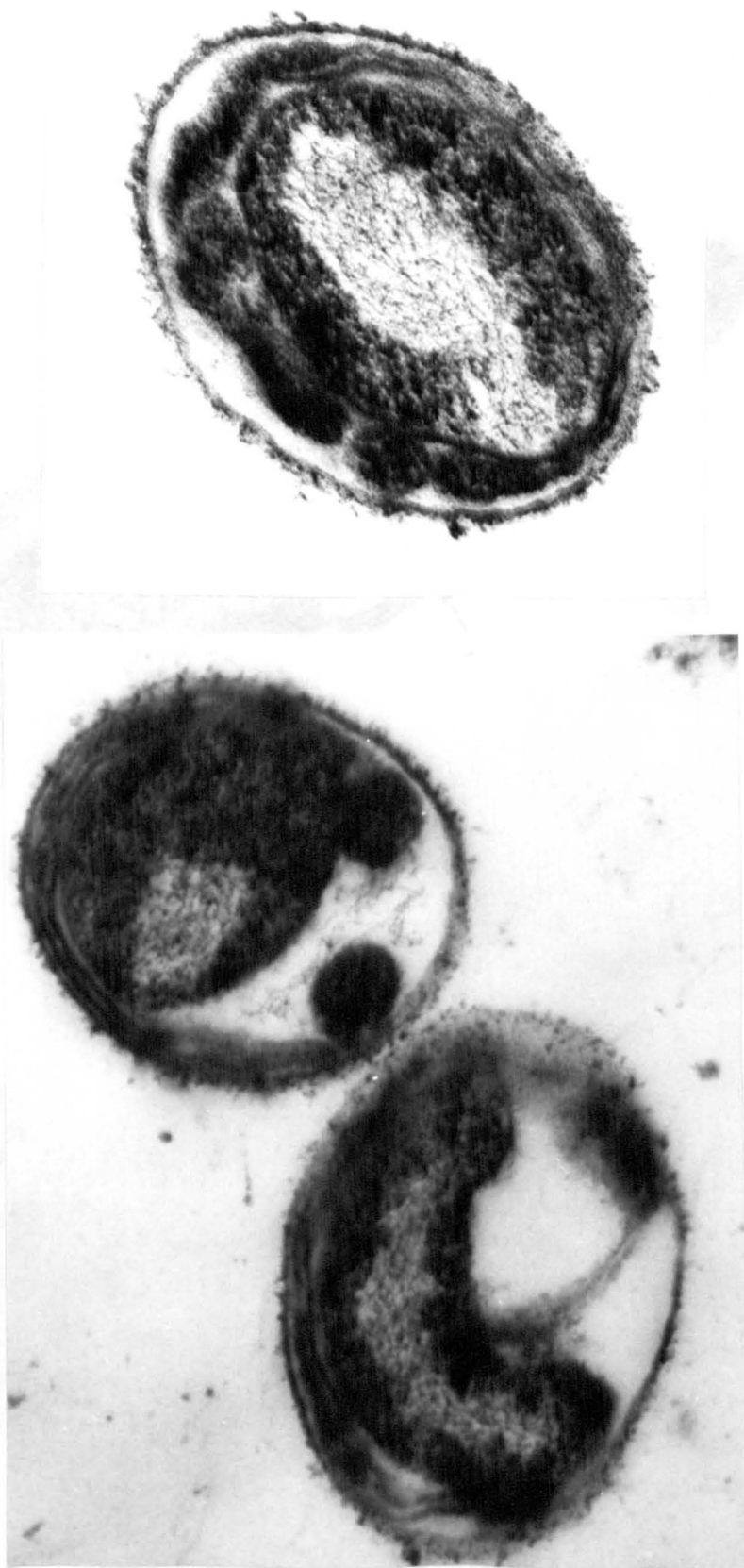


Plate 3.37 and 3.38

Cross section of developing vegetative cells of Rhodospirillum (Rm5) showing extensive compartmentalisation outwith the cell proper, i.e. between the cytoplasmic membrane and cell wall. (Kellenberger fixation)
(x 100,000)

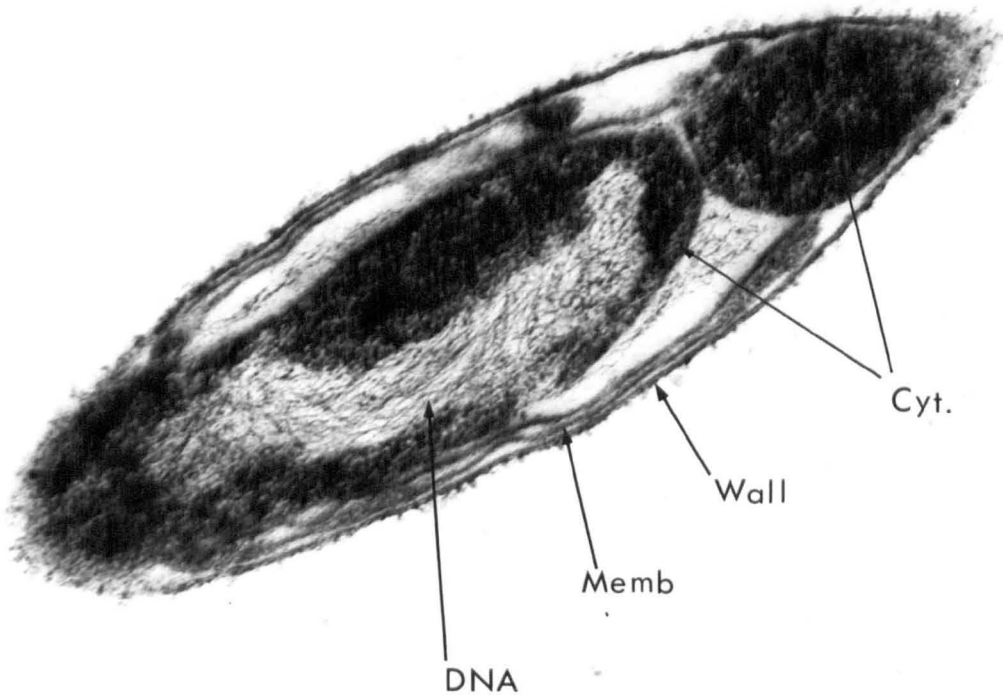


Plate 3.39

Transverse section of a developing vegetative cell showing the compartmentalisation of the mother cell and possible continuity of the daughter 'unit' with the filament. (Kellenberger fixation, x100,000).

Mother
cell

Plate 3.40

Transverse section of mother and daughter cells showing continuity of cytoplasmic 'unit' between them. (Kellenberger fixation x 60,000).

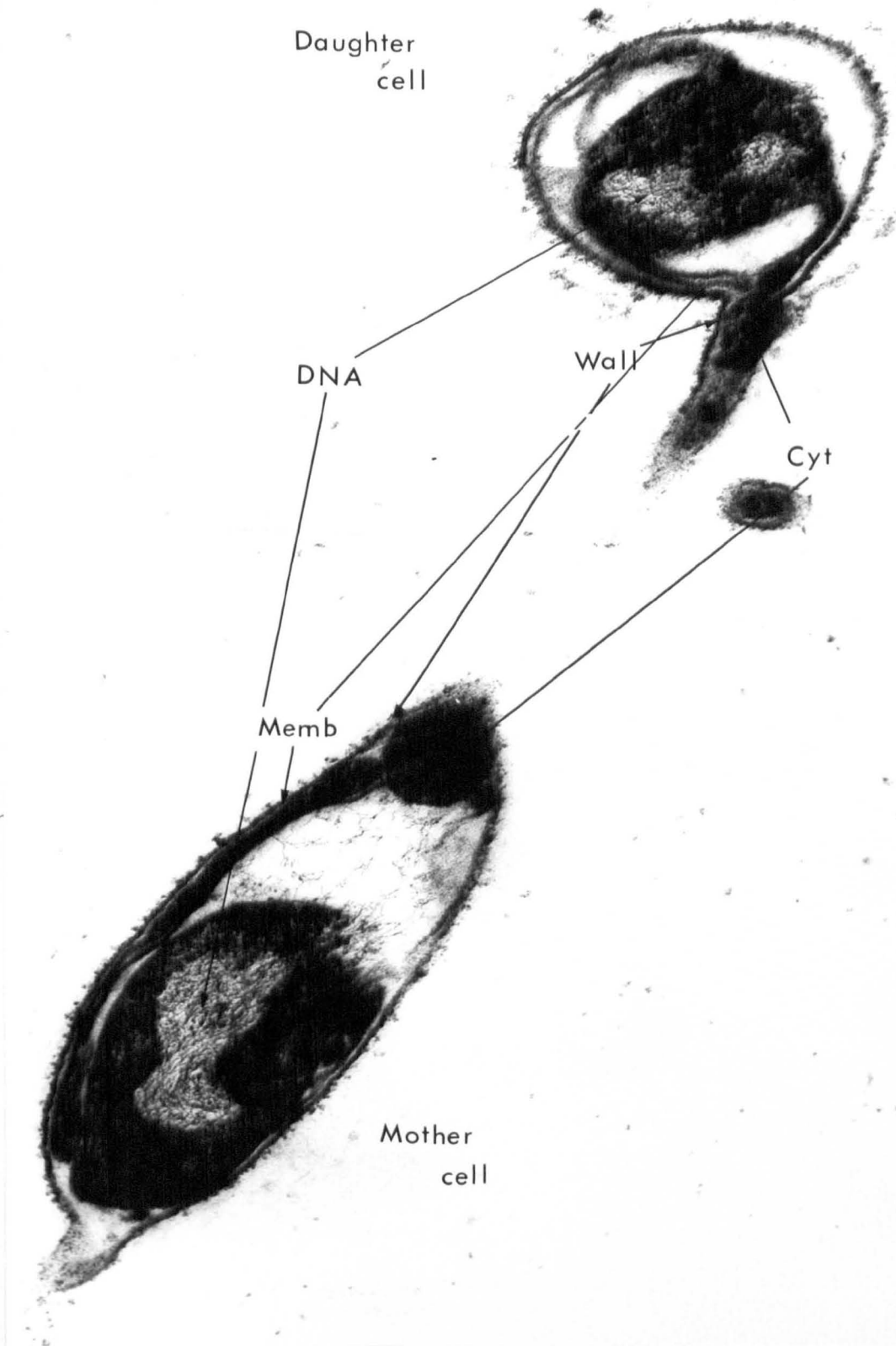


Plate 3.40

Transverse section of mother and developing daughter showing continuity of cytoplasmic 'unit' between both.
(Kellenberger fixation x 60,000).

volume cell wall, the daughter cell being largely preformed within the mother cell and enclosed in its own cytoplasmic membrane. That is to say the daughter cell is formed within the mother cell and extends into a filament as a functional unit, containing DNA, cytoplasm and cytoplasmic membrane but no developed lamellar system (figure 3.27,1-3). On completion of the first daughter cell 'unit', the mother cell starts a second reproduction process as indicated in figure 3.27,3, which when it encounters the constriction or plug (or possibly back pressures) from the first daughter cell, initiates cell wall synthesis and produces a side branch. There is in such a system an obligate requirement for cell wall or filament synthesis to be at the filament tip, or more correctly at the leading edge of the developing cell. Evidence for such comes from penicillin studies on growing cells (sphaeroplast formation, the position of which is indicative of the site of cell wall synthesis (Donachie and Begg, 1970) described in detail later, page 202) and a detailed analysis of viable systems, i.e. filament synthesis is a prerequisite for bud formation. There is a comparable obligate requirement for stalk or filament synthesis in Caulobacter and Hyphomicrobium prior to daughter cell formation (Moore and Hirsch, 1973; Staley and Jordan, 1973).

The above model retains the concept that the physiological and primary metabolic site is the cell body, i.e. the filament is relatively inert physiologically with the exception of the cell wall synthesising tip, no membrane or cytoplasmic elements require synthesis at this site since these are formed within the mother cell. The problem of DNA movement is solved as it moves simply with the cell

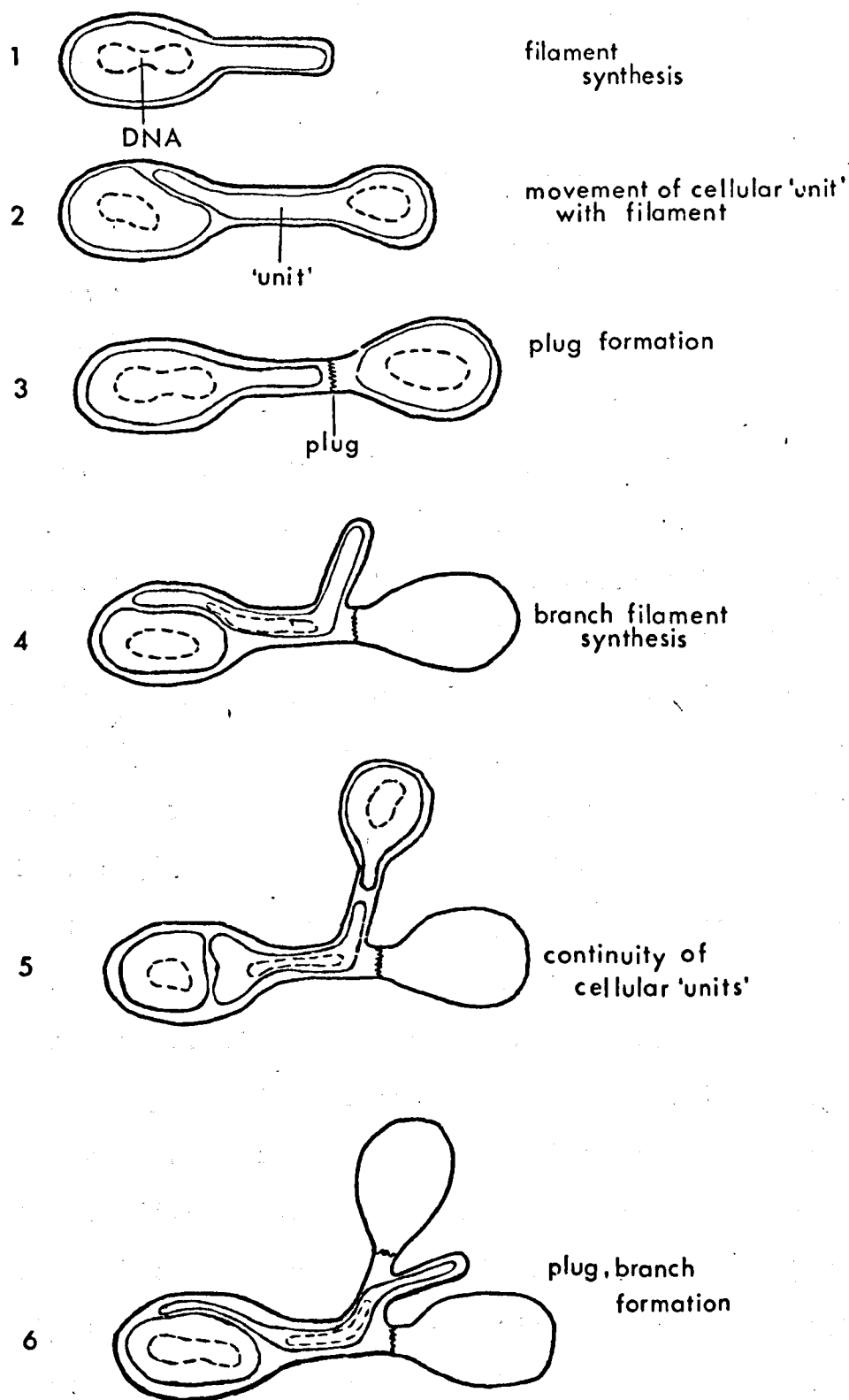


Figure 3.27

Schematic representation of the proposed reproductive cycle in Rhodomicrobium vegetative cells.

'unit', each cell 'unit' following on from the previous so retaining continuity in the system. This idea allows the mode of physiological replication of Rhodomicrobium to be identical to 'normal' bacterial growth and divisions with only one exception, the cell wall synthesis and subsequent cell division is displaced in time and relative positioning.

As described the model has taken into account the displacement or breach of the complex lamellar system during the sequence. From transverse and cross-section electron micrographs (plates 3.37-3.40) it is apparent that the cellular cytoplasm is 'packaged' within cytoplasmic membrane 'loops', as would be predicted from the proposed model (plates 3.39 and 3.40). Such compartmentalisation leads to the formation of periplasmic areas within the cell, i.e. regions interspaced between cytoplasmic membrane and cell wall which are necessary to accomodate the proposed movement of the cellular 'units'. These properties characteristic of all cells grown at high light intensities, may be a simple and efficient means of avoiding the lamellar membrane and disruption of the integrity of the mother cell 'unit' (figure 3.28). In addition no obstacles are encountered irrespective of the direction of growth; the filament tip is at this stage pre-destined to initiate filament synthesis from the tip not the base of the cell.

The growth pattern, ultrastructural and physiological details are all compatable with a system derived as above. However, to substantiate such a model detailed ultrastructural and cytological studies must be carried out on synchronised cell populations (section 4.3).

Figure 3.28

1. Mature swarm cell having complete and functional lamellar membrane complex. Initiation of reproduction i.e. DNA synthesis.
2. Extrusion of cytoplasmic membrane with filament synthesis. DNA movement.
3. Daughter cell (bud) formation. DNA movement with cytoplasmic membrane.
4. Completion of daughter cell 'unit'. Initiation of second cytoplasmic extrusion, utilising the periplasmic area to avoid the mother cell lamellar membrane system.
5. Second daughter cell 'unit' and filament synthesis encountered when daughter cell formation is bidirectional.

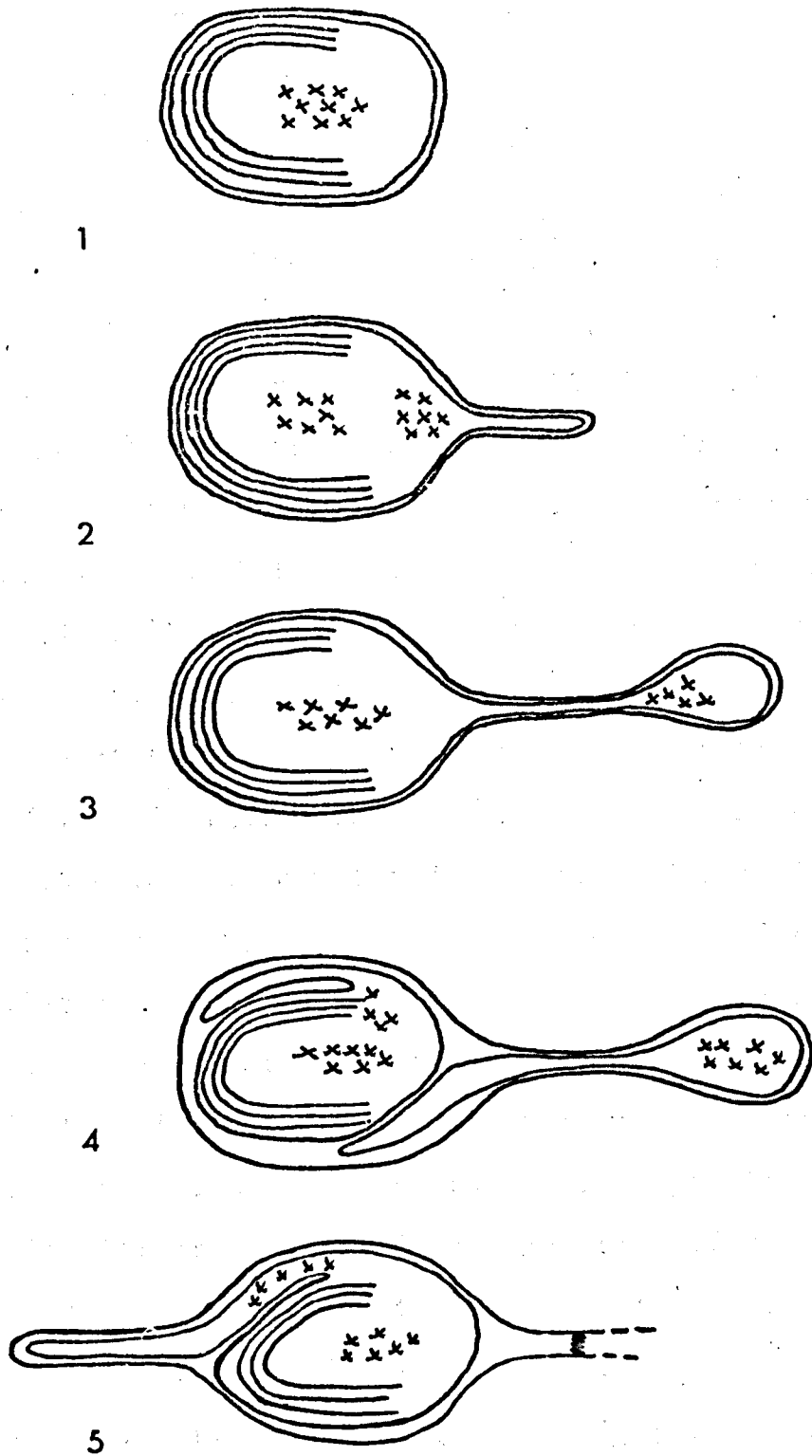


Figure 3.28

Diagrammatic representation of daughter cell formation avoiding disruption of the integrity of the mother cell 'unit'.

H) Ultrastructure of exospore germination.

The germination sequence of the exospore is not morphologically comparable with the growth sequence of vegetative cells. The exospore sequentially yields three to four daughter cells on filaments originating from the resting cell body. However, the ultrastructural concept proposed for vegetative cells is applicable to exospore outgrowth as well. Compartmentalisation is demonstrated in plates 3.41, 3.43, and 3.44 and bears a close relationship to that found in the vegetative system (plates 3.37 and 3.40). The internal ultrastructural organisation is, however, more complex. This is necessitated by the requirement to penetrate the extensive lamellar system.

Plate 3.41 is a transverse section of a germinating exospore which has completed formation of the first daughter cell (as judged by the formation of constriction c) and has initiated synthesis of the second daughter cell. (A schematic representation of this micrograph is given). Germination is characterised by apparent tubular incisions penetrating the membrane system to the cytoplasm-DNA complex. In this micrograph there is a suggestion of movement of DNA (presumably destined for the daughter cell) into the tubular structure. Additionally there would appear to be one insertion per filament (that of 1, plate 3.41; is below the plane of section). The insertions are membrane bound (plate 3.41, 3.42) and outwith the lamellar system (plate 3.45).

Detailed analysis of such micrographs reveals ultrastructural organisation similar to that proposed for vegetative cell growth with additional extension of the insertion

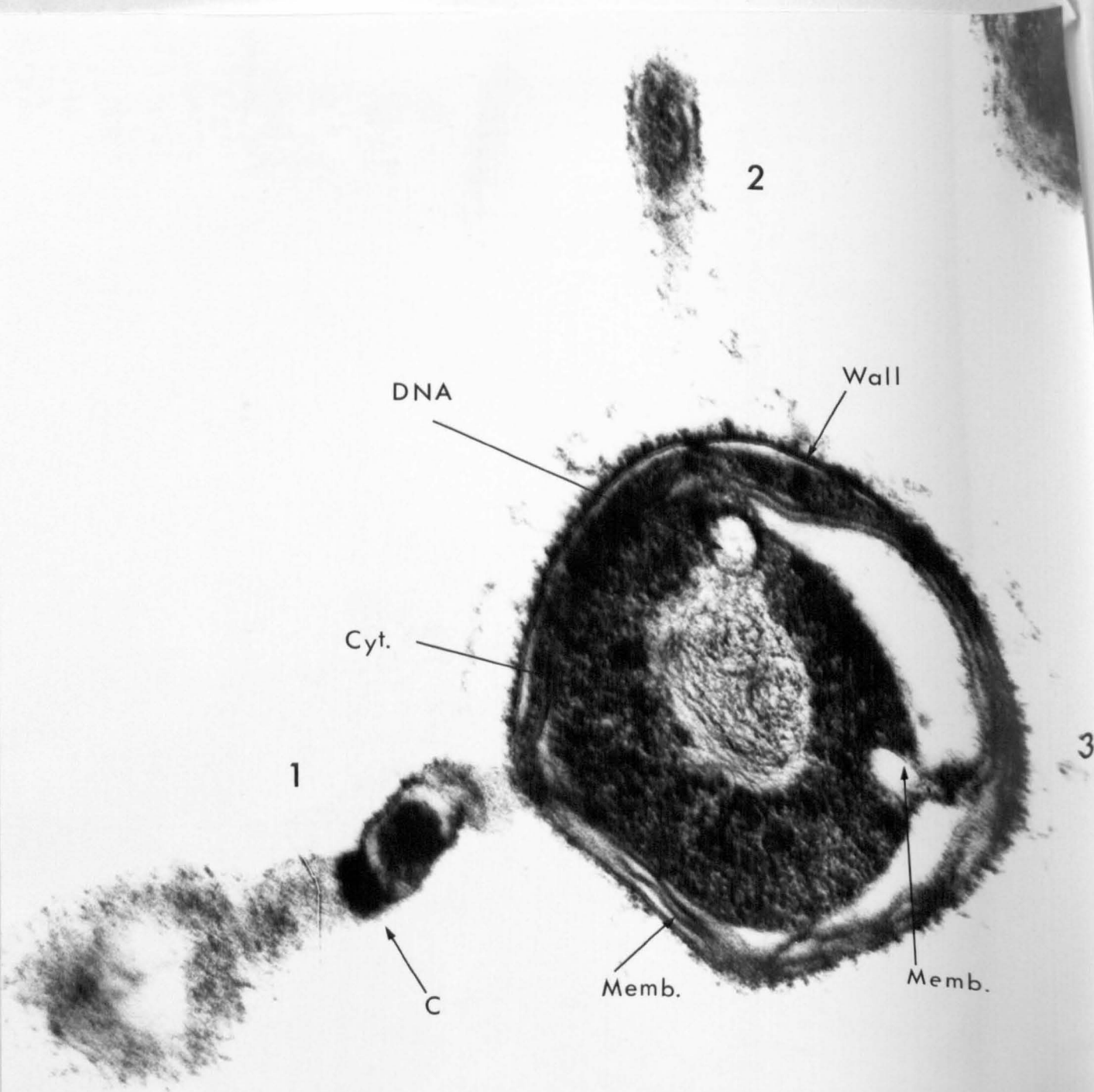
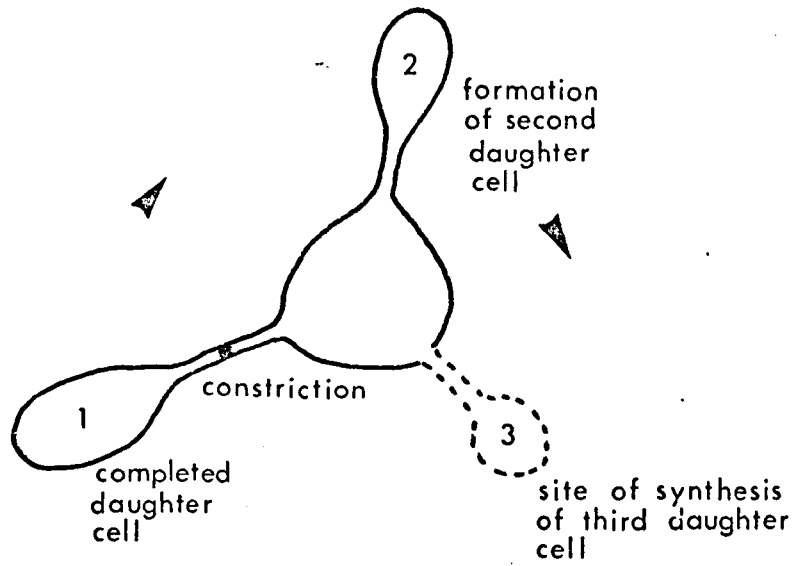


Plate 3.41

Transverse section of a germinating *Rhodomicrobium* (Rm5) resting cell. (Kellenberger fixation x 100,000)



Diagrammatic representation of the general morphology of the germinating exospore thin section shown opposite.

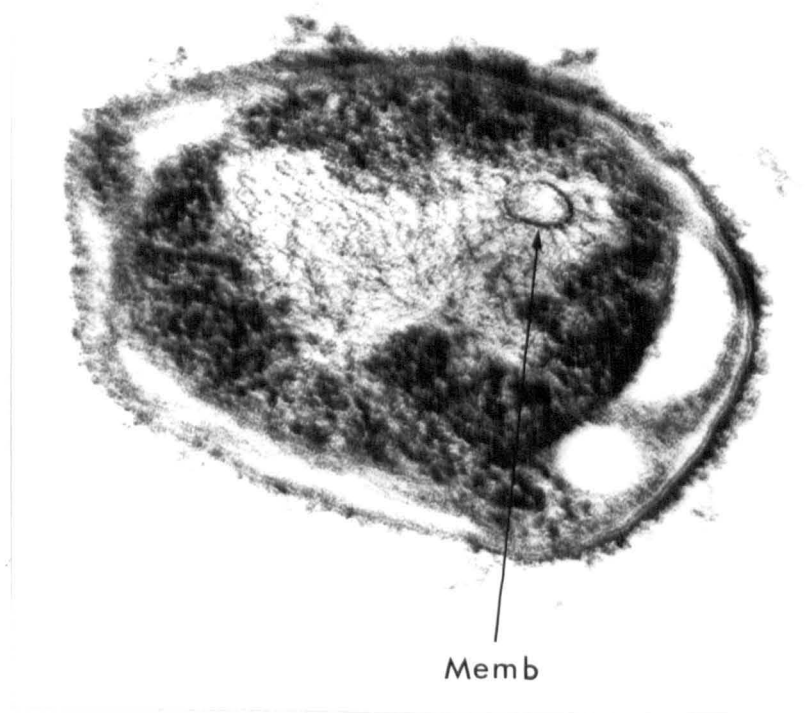


Plate 3.42

Transverse section of spore showing base of a membrane insertion (mi). (Kellenberger fixation x 100,000).

Plates 3.43 and 3.44

Transverse sections of exospores showing compartmentalisation.
(Kellenberger fixation x 100,000).

Plate 3.45

Cross section of Rhodomicrobium (Rm5) exospore showing depression
of the lamellar membrane system by the proposed tubular structure.
(x 100,000)



Plate 3.43



Plate 3.44

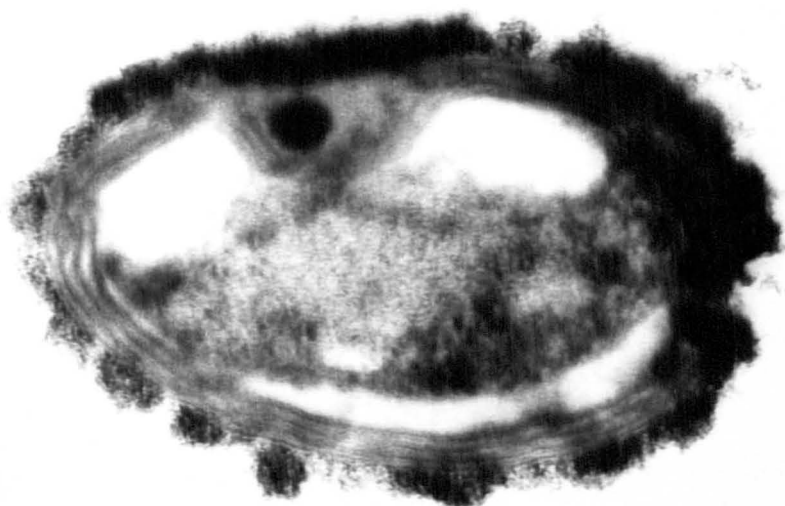


Plate 3.45

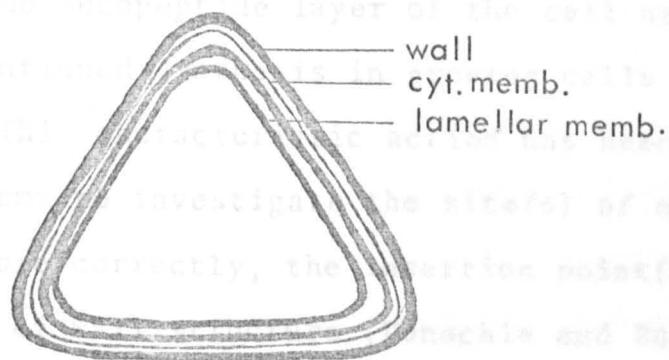
system (figure 3.29). As with swarm cell development these are preliminary studies and the above models are proposed as working hypotheses formulated from the information available.

I) Characteristics of the Rhodomicrobium filament.

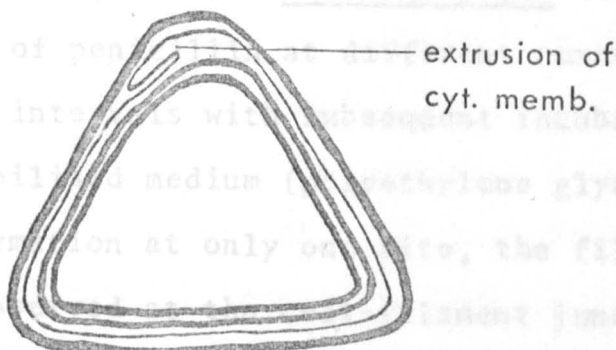
(i) Site of stalk and filament synthesis.

Autoradiographic pulse chase experiments performed by Schmidt and Stanier (1966) identified the site of Caulobacter stalk development as being at the junction of the stalk with the cell. Additionally they concluded from penicillin and lysozyme studies, that the stalk wall is a relatively inert non-growing structure. Corresponding studies on the filaments of Hyphomicrobium and Rhodomicrobium have not been documented. However, it has been suggested by Schmidt, (1971) that in Hyphomicrobium and by extrapolation in Rhodomicrobium, growth is from the base of the cell, i.e. at the junction of filament and cell. The present studies on Rhodomicrobium filament wall growth (autoradiography, enzyme and slide culture) do not conform with this model.

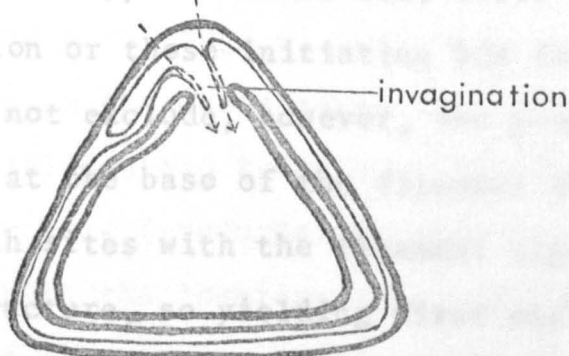
It is well established that the chemical site of lysozyme action is the β (1-4)-glycosidic link between N-acetylglucosamine and N-acetylmuramic acid units of the mucopeptide layer of the cell wall (Perkins, 1963). The enzyme in the presence of 0.001M EDTA took 20-30 minutes to show microscopic effect on the wall of Rhodomicrobium; both cell wall and filament walls were however equally effected. This observation substantiates the electron microscopic evidence that the murein layer of the cell wall is continuous with that of the filament.



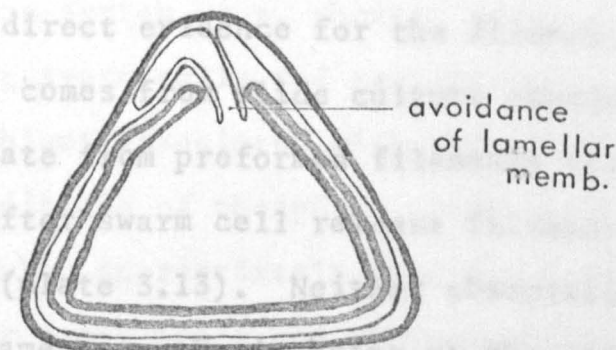
1



2



3



4

Figure 3.29

Diagrammatic representation of *Rhodomicrobium* exospore germination showing invagination of the cytoplasmic membrane giving rise to the observed tubular insertions.

In contrast to lysozyme, penicillin is not active in destroying the mucopeptide layer of the cell wall but inhibits its continued synthesis in growing cells (Murray, et al, 1965). This characteristic action has been exploited in several systems to investigate the site(s) of murein synthesis, or more correctly, the insertion point(s) (the growing points) of wall structure (Donachie and Begg, 1970; Higgins and Shockman, 1971). Rhodomicrobium cells incubated in the presence of penicillin at different concentrations for varied time intervals with subsequent incubation in osmotically stabilised medium (polyethylene glycol) showed sphaeroplast formation at only one site, the filament tip. No effect was observed at the cell-filament junction irrespective of the cell type examined i.e. cells initiating filament formation or those initiating bud formation. These observations do not exclude, however, the possibility of growth occurring at the base of the filament since growth may occur at both sites with the filament tip being the least stable structure, so yielding first and therefore relieving the pressure on the second growth point.

Further indirect evidence for the filament tip being the growth point comes from slide culture studies. Branch filaments originate from preformed filaments (plates 3.10 and 3.26), and after swarm cell release filament synthesis is re-initiated (plate 3.13). Neither observation can be explained by filament synthesis being at the junction of the cell and the filament.

Preliminary light microscope autoradiographic techniques may resolve the question, not only of filament synthesis but also of its physiological and functional capabilities.

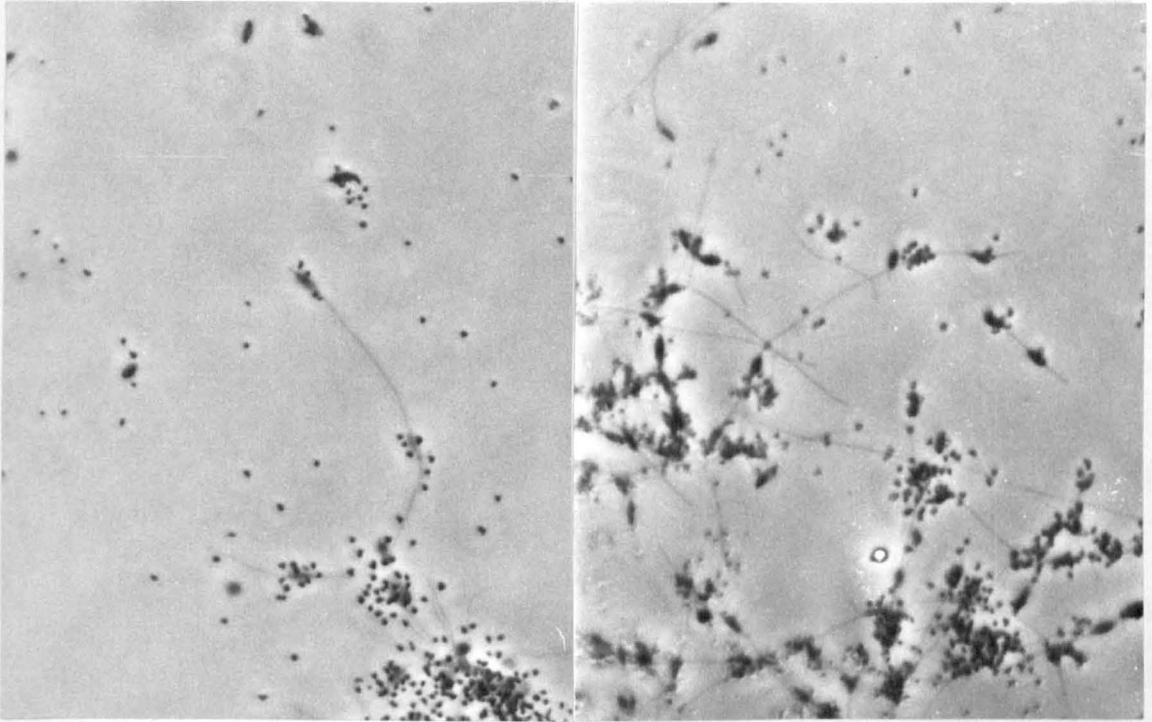
The autoradiographic procedure (section 3.2) was formulated using the uptake of L-(4-5-³H) leucine (to follow protein synthesis) in heterogenous photosynthetic cultures. Scatter and background emissions proved to be the most significant problems. However, these could be minimised by incorporating a phosphate limitation step in the experimental proceedings, i.e. the filament length could be increased or decreased as conditions demanded.

The initial experiments were limited in the context of filament synthesis and will undoubtedly only yield useful information when applied to synchronous cell systems (section 4.2). However, they did indicate the apparent inability of established filaments to incorporate or take up labelled amino acids revealing the cells to be centres of biochemical and physiological activity irrespective of their positioning in the chain sequence (plate 3.46).

This autoradiographic technique, employing 5-Amino (G-³H) laevulinic acid (precursor in chlorophyll synthesis) should indicate both the location and position in the temporal sequence of the cell cycle of synthesis of the photosynthetic membrane system, i.e. showing de novo synthesis within developing swarm cells and germinating spores. Additionally the problems associated with DNA movements, especially under conditions of phosphate shift up and shift down may be resolved with appropriately labelled material.

(ii) Caulobacter cross bands and Rhodomicrobium filament plugs.

The only structure found in the appendaged procaryotic genera which bears comparison with Rhodomicrobium 'plugs' are the crossbands found in the stalk of the Caulobacter family (Jones and Schmidt, 1973). Although the Caulobacter



Function of stalk Plate 3.46

Autoradiographs of Rhodomicrobium (Rm5) vegetative cells grown in the presence of L-(4-5-³H) leucine illustrating granule formation only in the vicinity of vegetative cells. (x 1100)

the attachment of Caulobacter by the stalk. The stalk of these microorganisms as a means of attachment to a solid surface source of nutrients in the form of organic matter. The stalk is considered by these microorganisms during their vegetative growth. The stalk is considered as a means of existence. Further, in this Poindexter considered the stalk to be a suspensory organelle in that it would maintain the non-motile cell at the air-surface interface, essential since Caulobacter are obligately aerobic. In contrast Tyler and Marshall (1957)⁸ viewed

stalk is not physically implicated in daughter cell formation the number of crossbands present has been shown to be directly related to the number of generations through which the cell has gone (Staley and Jordan, 1973), i.e. one crossband formed per replication cycle. The apparent solidity of the crossbands (Jones and Schmidt, 1973) would presumably dismiss any physiological function attributable to the stalk beyond the nearest crossband to the cell proper, solely because of the lack of continuity with the cell. Jones and Schmidt (1973) however considered these structures as physical supports for the stalk and membrane system. The one important ambiguity in this respect is the irregularity of spacing within the stalk, i.e. 3 to 4 crossbands may be found in 5 or 20 μ stalks. It may therefore be postulated that the common characteristic between Rhodomicrobium 'plugs' and Caulobacter crossbands is their obligate involvement in cell replication.

Function of cellular stalk and filament.

The integral cellular stalks of Caulobacter and the filaments of Hyphomicrobium and Rhodomicrobium have been the objects of much speculation as to their physiological function (Schmidt, 1971). Poindexter (1964) interpreted the attachment of Caulobacters by their holdfasts to other microorganisms as a means of establishing a continuous source of nutrients in the form of organic substances excreted by these microorganisms during their normal growth, i.e. ectocommensalism as a mode of existence. Further to this Poindexter considered the stalk to be a suspensory organelle in that it would maintain the non-motile cell at the air-surface interface, essential since Caulobacters are obligately aerobic. In contrast Tyler and Marshall (1967)^a viewed

the filament of Hyphomicrobium as a means of escaping encasement by manganese deposition and the holdfast as an anchoring device in turbulent freshwater environments. In a similar context the chain formations of Rhodomicrobium may be regarded as a means not of escaping encasement by deposition, but of escaping self-shading by other cells in the colonial system, itself necessary for efficient exploitation of aqueous niches by photosynthetic mechanisms.

A most impressive demonstration of environmental influence on Caulobacter stalk length was by Schmidt and Stanier (1966) who observed marked elongation of stalks by limitation of the available phosphate concentration. These studies gave rise to the idea that such cellular structures allowed a marked increase in the cell surface area so enhancing membrane-associated activities and nutrient uptake with a small increase in cell volume (Hirsch, 1968). Corresponding studies on Hyphomicrobium filaments showed a similar association of length with phosphate concentration. The idea of a structure varying in length in response to environmental stimuli is additionally attractive because all of the stalked or appendaged genera so far isolated are indigenous to freshwater where considerable variation in nutrient levels will be experienced. The question is therefore "is this an inducible system allowing maximum uptake with minimum cellular expenditure?". Schmidt (1971) claims that such is not the case since experiments failed to confirm that stalked Caulobacter cells can take up nutrients more efficiently than swimmers (non-stalked cells).

Although variation in phosphate concentration affects stalk or filament length, evidence is accumulating to indicate that variation in stalk length is a direct consequence of the growth rate, which in turn is influenced by the available phosphate. Evidence for this comes from studies on the effect of cyclic nucleotides and nucleoside triphosphates on Caulobacter stalks with the primary observation that variation in guanosine 3'5' monophosphate (GMP) concentration will invoke elongation or reduction in stalk length (Schmidt and Samuelson, 1972). In consequence the phosphate concentration may be regarded as influential on the nucleoside and nucleotide phosphate pools within the cell which in turn influences cell growth and replication eg. particularly with regard to DNA replication in Caulobacter which occurs only in stalked cells (Degnen and Newton, 1972). The stalk length of Caulobacter has also been found to be a function of the number of times the cell has reproduced (Staley and Jordan, 1973) i.e. one crossband is formed per generation. The intercrossband distances on the other hand reflect environmental parameters (figure 3.30). Within the context of growth rate being the principle effector of stalk or filament length, Dr. Harder (pers.comm.) has indicated that when Hyphomicrobium species are growing in a steady state condition, variation in growth rate, independent of phosphate concentration, invokes a filament response.

The above observations together with evidence presented below indicates that stalk or filament length is directly correlated with growth rate. The effect of phosphate concentration is therefore simply influential on this.

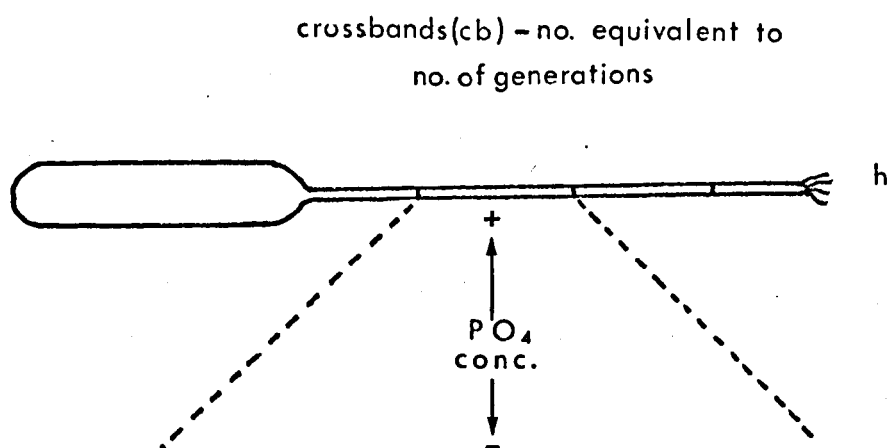


Figure 3.30

Schematic representation of a stalked Caulobacter cell showing crossband formation and the predicted segments ('unit') of variance in response to phosphate concentration (note such variance will only be possible during integration of 'unit' with cell proper).
(h, holdfast)

A close correlation between phosphate concentration and growth rate with concomitant increase or reduction in filament length can be shown in Rhodomicrobium. When grown under phosphate limitation colonial morphology is characterised by considerable intercellular distances (plate 3.47) with filaments attaining 15-20 times the length of the mother cell. Addition of inorganic phosphate ($0.1 \times 10^{-3}M$) to such cultures induces, within a short period, a considerable and marked increase in growth rate (figure 3.31). Studied on slide cultures such experiments revealed a burst of cell growth occurs in response to the addition of phosphate (as shown in plate 3.49). Initially under phosphate limitation filament length is extensive, on addition of phosphate there is maximum cell growth with very short filament lengths.

Control of the growth rate other than by inorganic phosphate levels was ineffective in yielding obvious morphological variation within the time course of growth of any particular culture. Individual carbon sources gave characteristic growth rates which were associated with characteristic and specific filament lengths. Variation in the concentration of these sources produced no effect on the growth rate, as would be expected, nor any change in filament length. These alterations only extended the period of exponential growth. Similarly with variation in the nitrogen source and concentration. Although changes in light intensity (50-1000 lux) resulted in variation of the growth rate, cellular morphology remained relatively constant. Thus accurately controlled and consistent marked

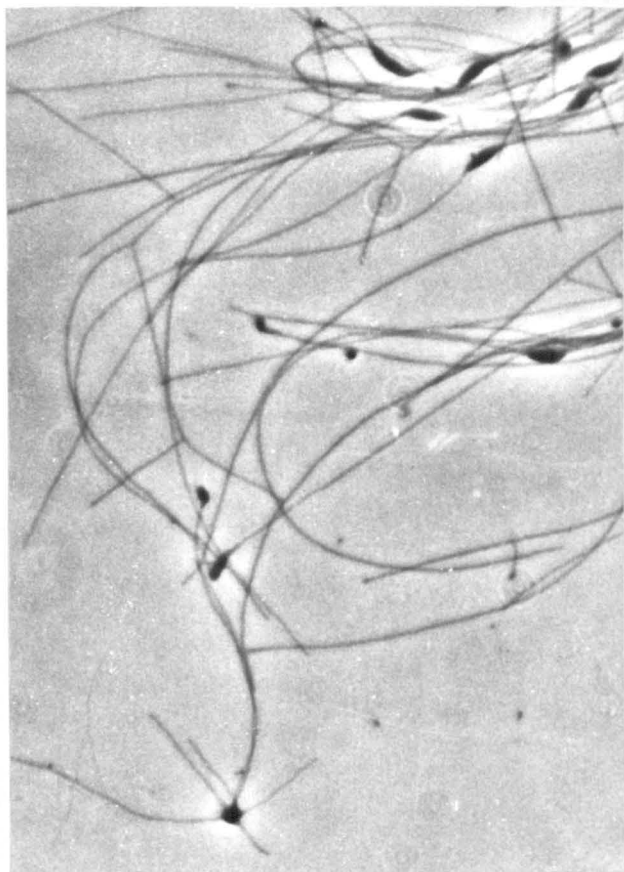
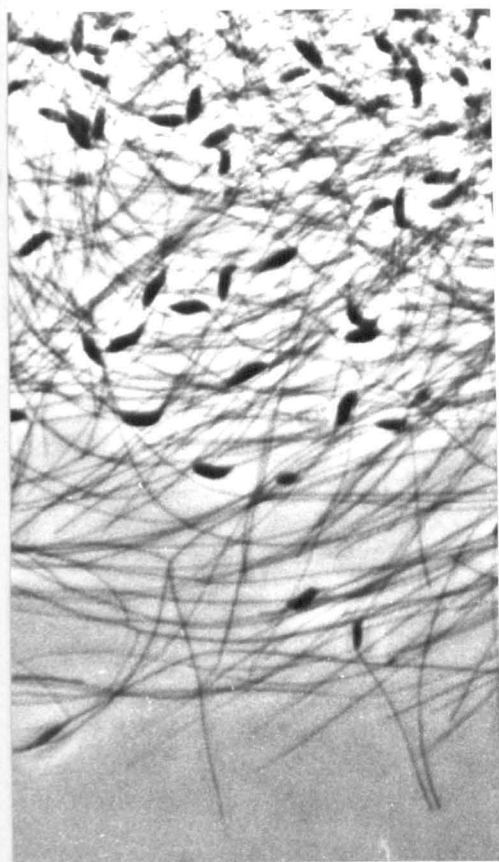


Plate 3.47

Rhodospirillum (Rm5) vegetative cells growing
on phosphate limited medium. (Phase contrast x 1250)

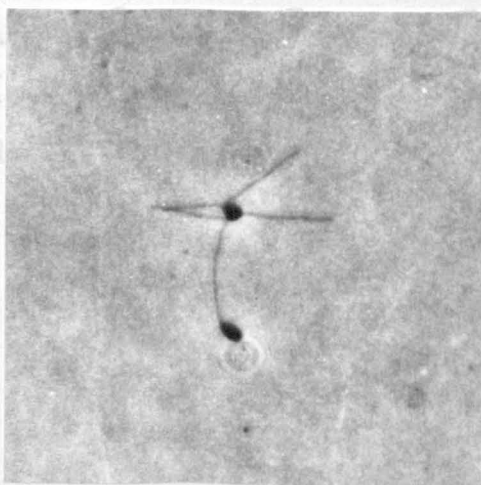
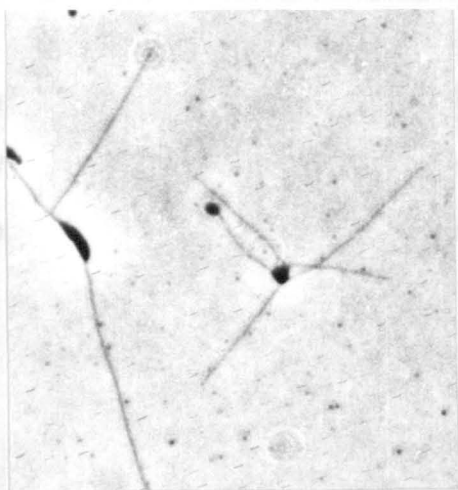


Plate 3.48

Rhodospirillum (Rm5) resting cells germinated
in medium low in phosphate concentration.
(Phase contrast x 1250)

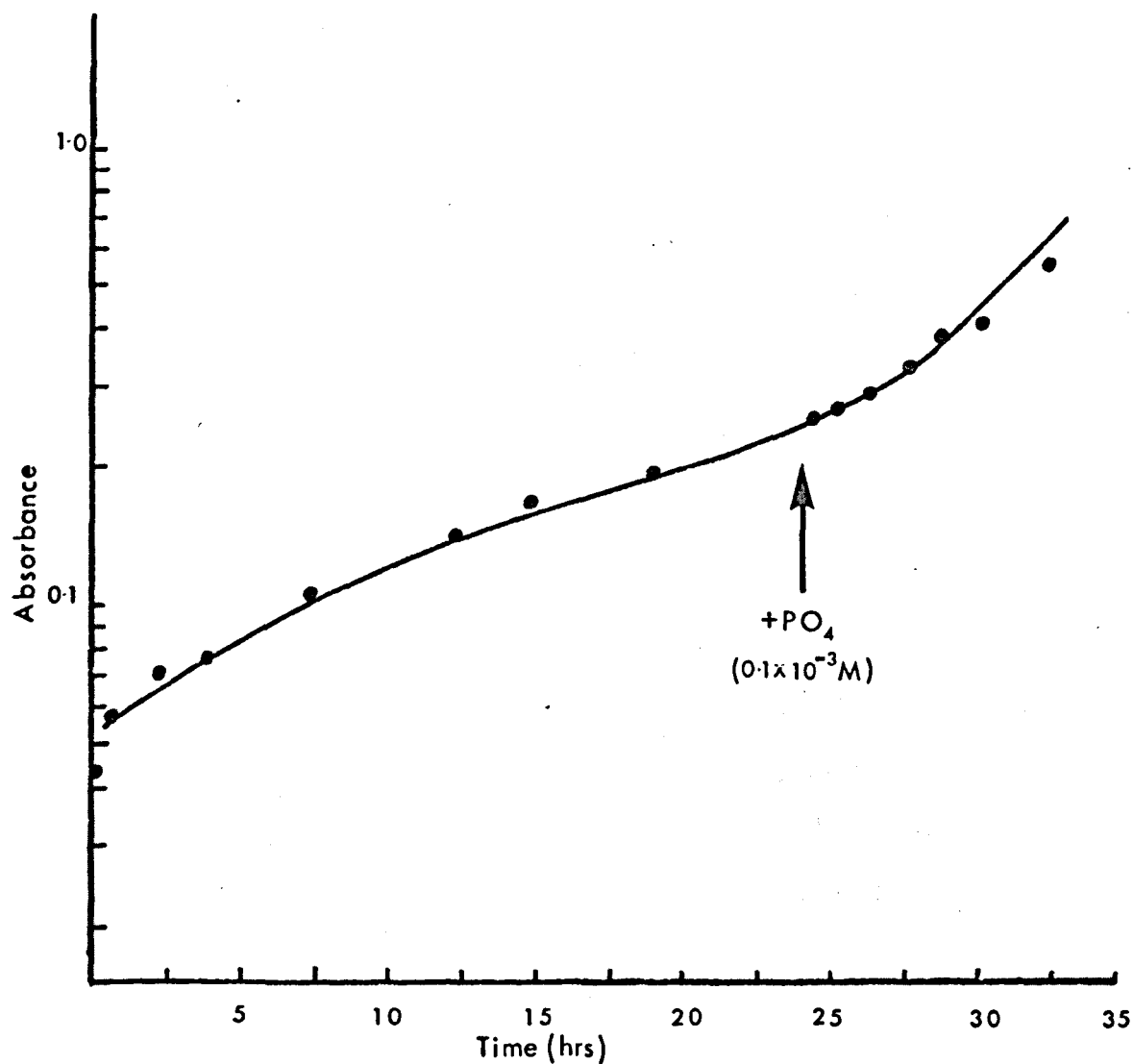


Figure 3.31

Growth curve of Rhodomicrobium (Rm5) grown initially on low phosphate with subsequent addition of inorganic phosphate to a concentration of $0.1 \times 10^{-3} \text{M}$.

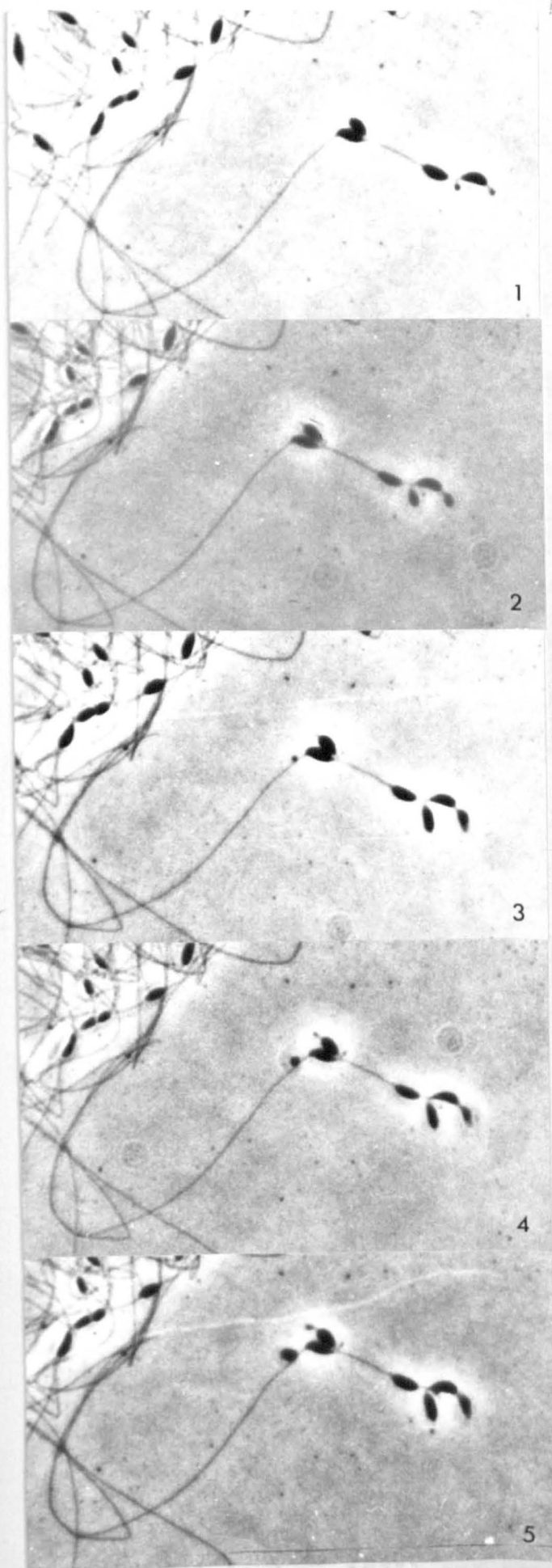


Plate 3.49

Vegetative cells of
Rhodomicrobium (Rm5)
 initially grown on low
 phosphate levels and then
 subjected to a concentration
 of $0.1 \times 10^{-3} \text{M}$.

(Phase contrast x 1250)

variation of filament length could be achieved only by variation in the inorganic phosphate concentration expressed as a change in the growth rate, possibly by influencing DNA replication. It should be noted, however, that all these results are characteristic of batch culture growth - not steady state systems.

Failure to recognise the morphological variations of this nature has resulted in the misidentification of appendaged procaryotic organisms observed directly in natural samples. Mirsch and Rheinheimer (1968) for instance, have fallen into this trap. They 'identified' an organism as a strain of the genus Ancalomicrobium. In fact the organisms they describe are clearly Rhodomicrobium exospores germinating in freshwater low in nutrients (correspond closely to plate 3.48).

Questions about the function of filaments have several plausible answers. Such as, that they are a means of replication imposed on the organism by the complex lamellar membrane system, and/or that they are the basis of a colonial system which has survival advantage in aqueous environments, and/or (doubtfully) that they increase the surface area of the cells so aiding nutrient uptake in dilute nutrient conditions. All notions have evidence for and against them. It is of course possible that these cellular extensions may serve multiple physiological functions. The characteristics of import, however, are the association of filament length with growth rate and the obligatory synthesis of filament prior to cell formation. The latter finding has an analogy with stalk synthesis and cell production in Caulobacter (Staley and Jordan, 1973) and Hyphomicrobium (Moore and Mirsch, 1973).

From these observations it becomes apparent that two primary functions of the filament of Rhodomicrobium are:

- a) Necessity for cell replication by avoidance of the lamellar membrane system.
- b) Colonial integration and efficient exploitation of favourable ecological nichés.

It is however, difficult to examine the organism and establish a hierarchy of physiological or ecological functions applicable to the filament, primarily because of the integraty of the system. The organism has 'evolved' and become orientated to efficient exploitation of aqueous environments. There is the expression of colonialism (chain forming vegetative cells), dipersal (swarm cells), and survival (exospore formation). The variance in cell 'type' is modelled to adaptation of the organism to the variances experienced in aqueous environments.

J) Rhodomicrobium bacteriophage.

No bacteriophage active on Rhodomicrobium has yet been reported in the literature. However, bacteriophage have been obtained for Hyphomicrobium (Gerencser and Voelz, 1971; Voelz et al, 1971), Caulobacter (Boindexter, 1964; Schmidt, 1966; Agabian-Keshishian and Shapiro, 1971; Jollick, 1972) and Rhodopseudomonas palustris (Bosecker, et al, 1972).

All of these genera are attractive models for studying differentiation (obligate reproductive cycle). Therefore bacteriophages may prove, and in the case of Caulobacter (Shapiro et al, 1971) have proved, to be useful tools in this context. Their value lies not only in their potential for analysis of various genotypes of modified life cycles but in the specificity of adsorption and replication within

the temporal sequence of the differential cell cycle.

Enrichments using chloroformed sewage and pond water inocula for bacteriophage showing specificity toward Rhodomicrobium consistently lysed the bacterial culture within 2 weeks (incubation at 30°C in the light). The cell specificity of the bacteriophages active on Hyphomicrobium, Caulobacter and Rh. palustris suggests that bacteriophage of Rhodomicrobium might be specific for only one cell type in the differential sequence. Therefore 'phage isolations were performed on developing swarm cell populations. By this procedure a few phage plaques, varying in diameter from 1-2mm, were obtained. Inoculation, from these plaques, into 2 litre Rhodomicrobium liquid cultures repeatedly produced lysis within 8-10 days. Precipitation of the lysate by sodium chloride, polyethylene glycol treatment and centrifugation of the precipitate on a step CsCl gradient produced a characteristic 'phage band. The infectivity of this preparation was however, inconsistent and very low i.e. 'phage plaques could not be obtained consistently nor could they be propagated outwith the above procedure.

Electron microscopic examination of the culture lysate and the purified 'phage band revealed the presence of only one 'phage type (plate 3.50), which is comparable in general morphology to bacteriophage T₂ (plate 3.50). The most distinctive morphological difference is the characteristic structure of the Rhodomicrobium phage tail.

The adsorption site(s) and replication of the 'phage cell are as yet undetermined. However, the apparently low infectivity suggests high specificity which can be explained as particular receptor sites being present during a limited period of the cell cycle.

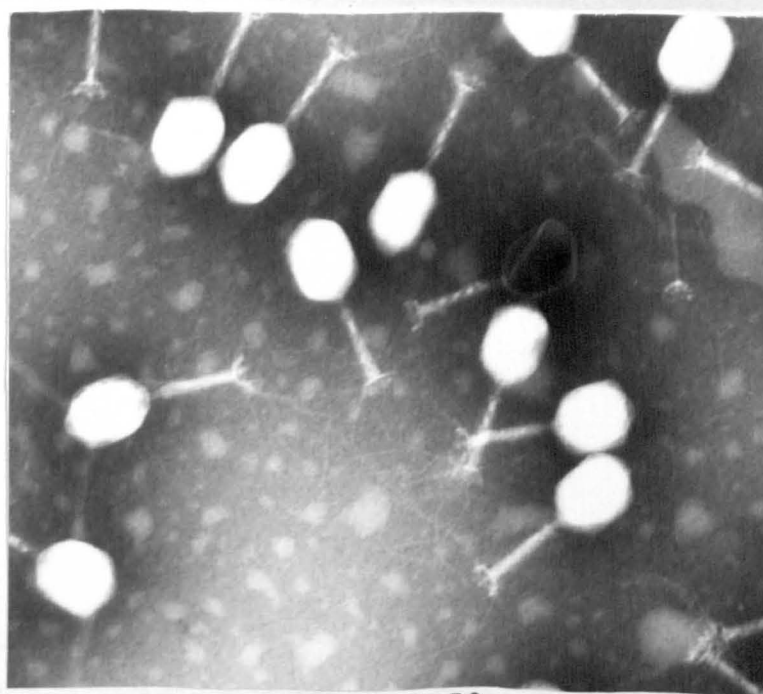
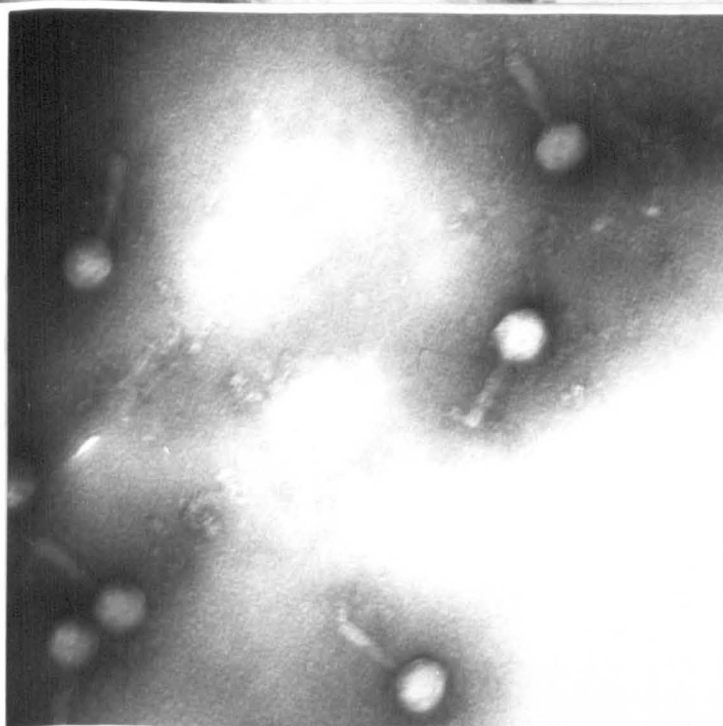
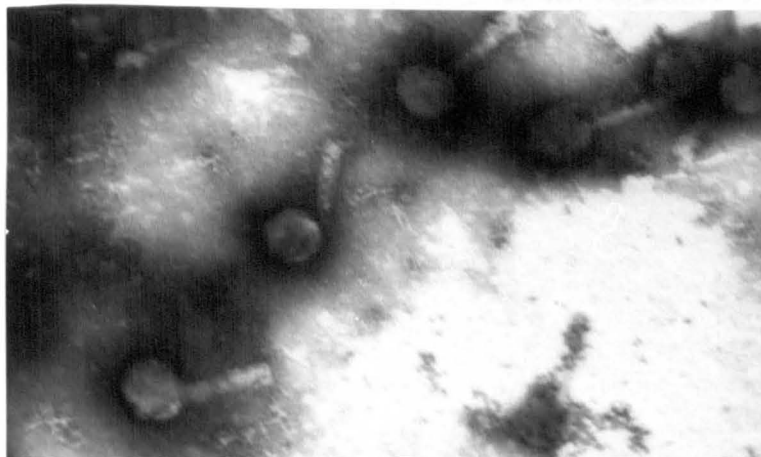


Plate 3.50

Phosphotungstic acid (1% w/v) negatively stained electron micrographs of a Rhodospirillum bacteriophage in comparison to bacteriophage T2.

Section 4.

Characterization and exploitation of a model
differentiation system using the budding appendaged
photosynthetic bacteria:

Rhodopseudomonas acidophila, Rh. palustris and Rhodomicrobium.

Section 4. Introduction.

Characterization and exploitation of a model differentiation system using the budding appendaged photosynthetic bacteria:

Rhodopseudomonas acidophila, Rh. palustris and Rhodomicrobium.

The recent rapid advances in genetics, biochemistry and molecular biology have resulted largely from the use of microorganisms as experimental systems. This approach was justified by the assertion that the basic biochemical structure and reaction mechanisms needed for cell reproduction evolved at the single cell stage. The coding mechanisms of transcription and translation and the biochemical pathways of different organisms are remarkably similar, although alternate pathways and multiple enzymes allow a certain variation.

With respect to differentiation such a unifying concept is usually not advanced, presumably because differential tissues look so different from one another. Undoubtedly the ultimate differentiation into highly specialised tissue requires very specific biochemical reactions which can be found only in particular cell types. Nevertheless, the essential features of the initial reactions, converting growing 'general purpose' cells into 'specialist' cells may be quite similar in pro- and eucaryotic organisms.

The study of differentiation in microorganisms has advantages not only of those commonly recognised in microorganisms eg. ease of manipulation, simplicity of growth systems and definition of the chemical environment, but includes the possibility that many developmental mutants,

blocked at different stages of development, can be isolated. Attempts can then be made to analyse the biochemical deficiencies and correlate these with morphological alterations. In addition the mutations can be genetically mapped allowing the determination of the number of genes required for each developmental stage.

Few bacteria undergo clearly defined morphogenetic changes other than cell division in their life cycle. However, the structural and physiological changes during development of one group of microorganisms, the aerobic spore formers, has been intensively studied and reviewed (Fitz-James and Young, 1969; Halvorson, 1965; Hanson et al, 1970; Holt and Leadbetter, 1969; Mandelstam, 1969; Schaeffer, 1969). The information available on the sporulating bacilli as a model system of cellular differentiation has been documented by Freese (1972) and although such a model will continue to be successfully exploited the specialised nature of spores restricts their value in as much as the initiation of, or commitment to differentiate is not an obligate step within the life cycle (figure 4.1) but an occasional, environmentally stimulated event. Consequently there has been a search for new model systems offering insight into other facets of differentiation not catered for by the sporulation or germination cycle of the endospore formers.

A major disadvantage of the procaryotic differentiating model systems concerns the unproven validity of extrapolating procaryotic to eucaryotic events. Not all eucaryotic cells are independent, self contained reproductive agents but 'interactors' with their surroundings including other cells and their products. Few cells or cell groups can be envisaged as differentiating on their own. There are procaryotic

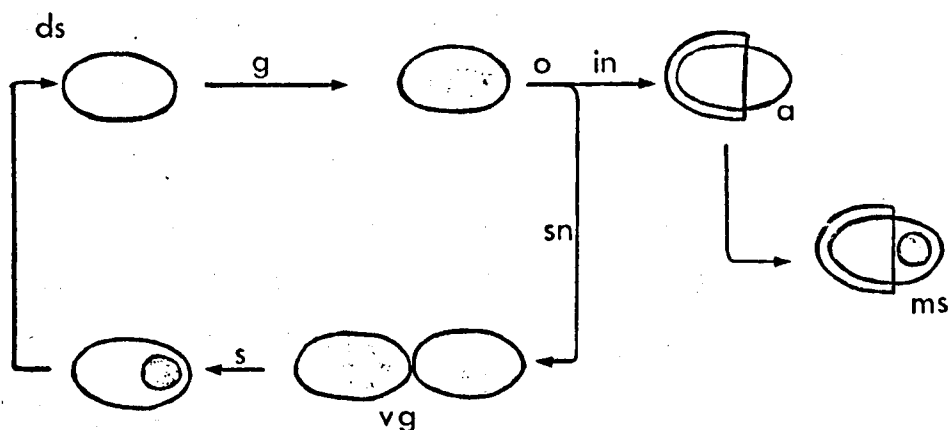


Figure 4.1

Life cycle in Bacillus.

ds	Dormant spore
g	Germination
o	Outgrowth
in	Outgrowth + insufficient nutrients
a	Arrested outgrowth
ms	Microcycle sporulation
sn	Sufficient nutrients
vg	Vegetative growth
s	Sporulation.

(Hanson, Spielgelman, Halvorson, 1970)

cellular systems which meet one or other of these parameters but none contain all in one system.

Microorganisms, other than the endospore formers, which offer possibilities for study in the desired context of differentiation are the blue green algae, Streptomyces, Arthrobacter, Myxobacter, the cellular slime moulds and Caulobacter.

Heterocyst formation in blue green algae is an obvious example for exploitation (Carr and Bradley, 1973) in that there are both marked morphological and physiological alterations from the vegetative cell eg. N_2 fixation and loss of photosystem II. However, the difficulty of obtaining preparations of cell populations of one cell type, and the lack of a synchronising procedure is a severe handicap. Streptomyces spp. can be grown in defined media and are particularly amenable to genetic study (Hopwood and Sermoniti, 1962) but suffer from a lack of biochemical definition of the differentiation process. On the other hand Myxobacteria (Dworkin, 1973) (figure 4.2) and the cellular slime moulds (Garrod and Ashworth, 1973)(figure 4.3) give not only cell to cell differentiation but can also be used as a model system for the investigation of cell-cell interactions, a property not expressed by the other models, with the exception of the blue green algae which form heterocysts at intervals along filaments to form a precise pattern (Mitchison and Wilcox, 1972). In contrast to the above, Arthrobacter presents a simple differentiation process which consists of a biochemically and morphologically well defined sphere-rod transition which is under environmental control (Ensign and

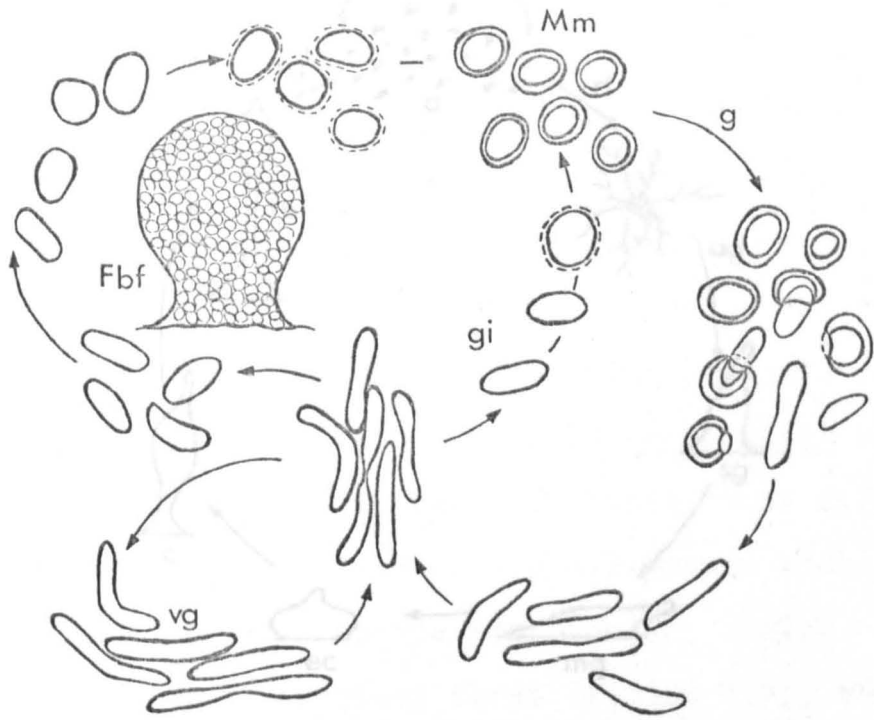


Figure 4.2

The life cycle of Myxococcus xanthus.

discoideum.

- Mm Mature myxospore
 g Germination
 gi Glycerol induction
 vg Vegetative growth
 Rbf Fruiting body formation.

ag Aggregation
 sg Standing grow.
 (Dworkin, 1973)
 mg Migrating grow.

ec Early culmination.

c Culmination

fb Fruiting body

sg' Spore germination

(Garrod and Ashworth, 1973)

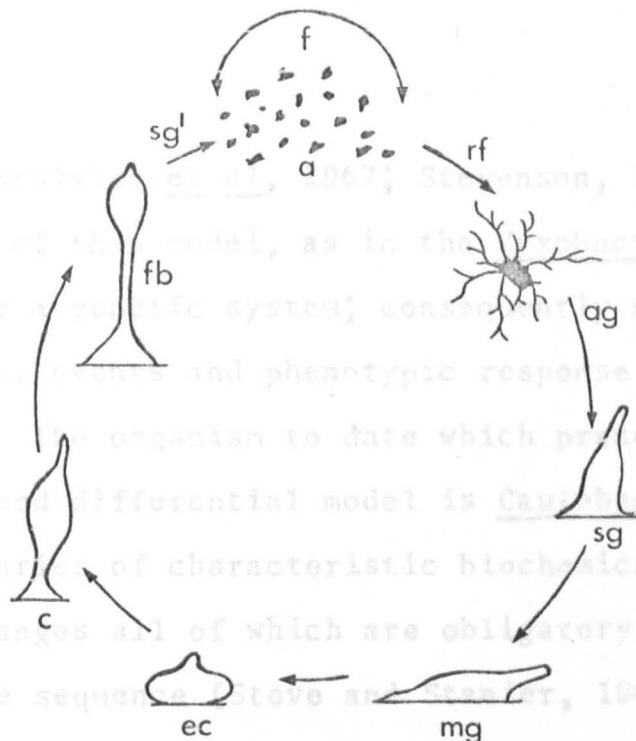


Figure 4.3

Life cycle of the cellular slime mould Dictyostelium discoideum.

- a Amoebae
- f Food
- rf Removal of food
- ag Aggregation
- sg Standing grex.
- mg Migrating grex.
- ec Early culmination.
- c Culmination
- fb Fruiting body
- sg' Spore germination

(Garrod and Ashworth, 1973)

Wolfe, 1964; Krulwich et al, 1967; Stevenson, 1968). The main drawback of this model, as in the Myxobacteria, is the absence of a genetic system; consequently no relation between genetic events and phenotypic response can be demonstrated. The organism to date which presents the simplest defined differential model is Caulobacter which undergoes a series of characteristic biochemical and morphological changes all of which are obligatory within a regulated time sequence (Stove and Stanier, 1962).

The three morphological forms (figure 4.4), the growth pattern, the polarity of cell development and asymmetric division of Caulobacter illustrate that growth of this organism is composed of two related cell cycles the dividing cells of which are morphologically and genetically identical. However, cell growth and metabolism must be regulated to accomodate the extra time required for stalk development (cell maturation) and it is this obligatory event which has focused attention on this system i.e. the obligate temporal sequence of events.

The major factor governing the value of a model differentiating system is the quantitative and qualitative aspects of cell synchrony. In endospore formers synchronous sporulation and germination is now relatively easily achieved (although not without application of a physiological shock) and has been extensively reviewed (Sussman and Holvorson, 1966; Keynan and Evenchik, 1969; Levinson and Hyatt, 1969). However, synchronisation of non-sporing genera, outwith those which have inherent synchrony in their life cycles eg. Myxobacteria, Geodermatophilus (Ishiguro and Wolfe, 1970) and the cellular slime moulds, has posed

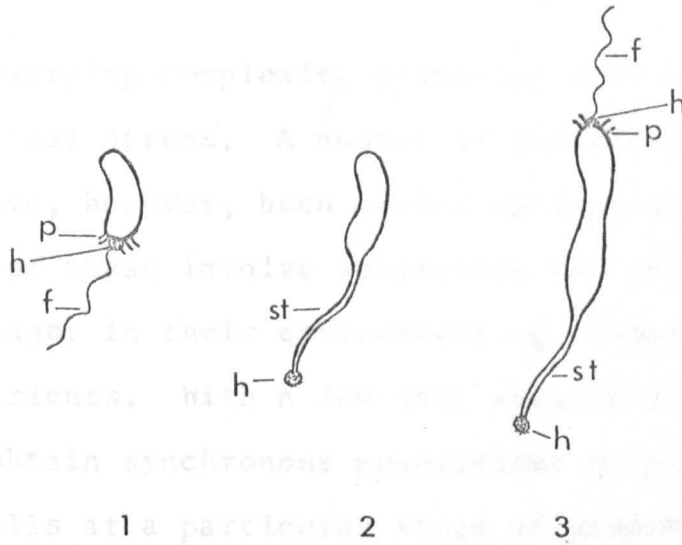


Figure 4.4

Principle developmental stages of Caulobacter sp.

1. Motile swarm cell.
2. Stalked mother cell.
3. Mother-daughter cell.

p Pili
h Holdfast
f Flagellum
st Stalk

been well documented (figure 4.3) (Whittenbury, 1967; Newton et al., 1971; Degnen and Newton, 1972). Populations of this organism, although showing spontaneous differentiation in a fixed temporal sequence, are amenable to analysis by the established techniques of bacterial genetics, are only now beginning to be analyzed at the molecular level.

The role of transcription in the temporal control of development of Caulobacter has been investigated by Degnen (1972) using rifampin, a specific inhibitor of the transcription

problems of varying complexity, primarily with selection and physiological stress. A number of synchronisation techniques have, however, been worked out (Helmstetter, 1969). The majority of these involve subjecting the cells to a series of changes in their environment eg. temperature, light and nutrients. With a few cell systems it has been possible to obtain synchronous populations by physically separating cells at a particular stage of growth (Helmstetter and Cummings, 1963; Mitchison and Vincent, 1965). The most promising of the separation techniques is the use of differential gradient centrifugation which has been successfully employed with Caulobacter (Shapiro et al, 1971) and Rh. palustris (Whittenbury and McLee, 1967; Westmacot, 1973 pers. comm.). However, to avoid possible physiological shock arising from differential centrifugation, Degnen and Newton (1972) have developed a system for obtaining synchronous swarm cell populations of Caulobacter by exploiting the adhesive properties of the holdfast.

Characterisation of the major physiological and morphological events of the Caulobacter life cycle have been well documented (figure 4.5) (Poindexter, 1964; Shapiro et al, 1971; Degnen and Newton, 1972). Synchronised populations of this organism, although showing morphological differentiation in a fixed temporal sequence which is susceptible to analysis by the established techniques of bacterial genetics, are only now beginning to be exploited at the molecular level.

The role of transcription in the temporal control of development of Caulobacter has been investigated by Newton (1972) using rifampin, a specific inhibitor of the initiation

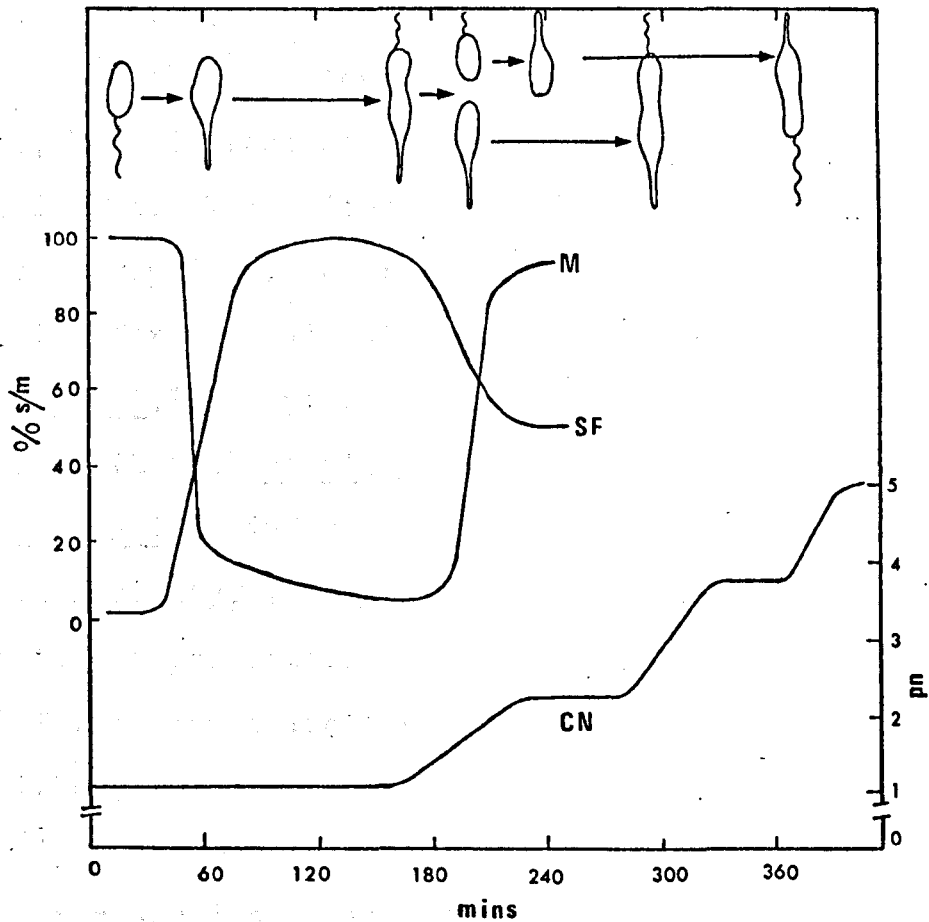


Figure 4.5

Growth and development of Caulobacter crescentus in synchronous cultures of swarm cells.

%SM % stalked or motile cells.

M Motility.

SF Stalk formation.

pn Particle number.

CN Cell number.

(Newton, 1972)

of transcription (figure 4.6). The implications of his findings are that development in Caulobacter is controlled, in part, by differential gene expression at the level of transcription. This situation therefore shows some analogy with transcriptional control in Bacillus subtilis where synthesis of ribosomal RNA is shut off during sporulation (Hussey et al, 1971) presumably by an alteration in the RNA polymerase (Losick et al, 1970). Similarly in Bacillus amyloliquefaciens infected with ϕ 29 bacteriophage where the specific activity, template specificity, stability and sedimentation properties of the RNA polymerase differ from those of the enzyme isolated from non-infected cells (Holland and Whitley, 1973). The study of such regulatory mechanisms, transcriptional, post-transcriptional or translational, in Caulobacter species is simplified because the developmental steps are so well defined and obligatory.

Rhodomicrobium, Rh. palustris and Rh. acidophila all possess life cycles which are similar in some ways to that of Caulobacter sp. i.e. obligate dimorphic life cycles involving the formation of filamentous mature mother cells from motile swarm cells. Additionally these organisms, all members of the Athiorhodaceae, form a differential gradient of development (table 4.1); the degree of morphological and possible physiological differentiation increasing dramatically from Rh. acidophila through Rh. palustris to Rhodomicrobium. This characteristic is unique to this group of organism. As a consequence of the physiological and morphological diversity and the differential developmental gradient, a method was devised for obtaining synchronous swarm cell populations. The value of these microorganisms

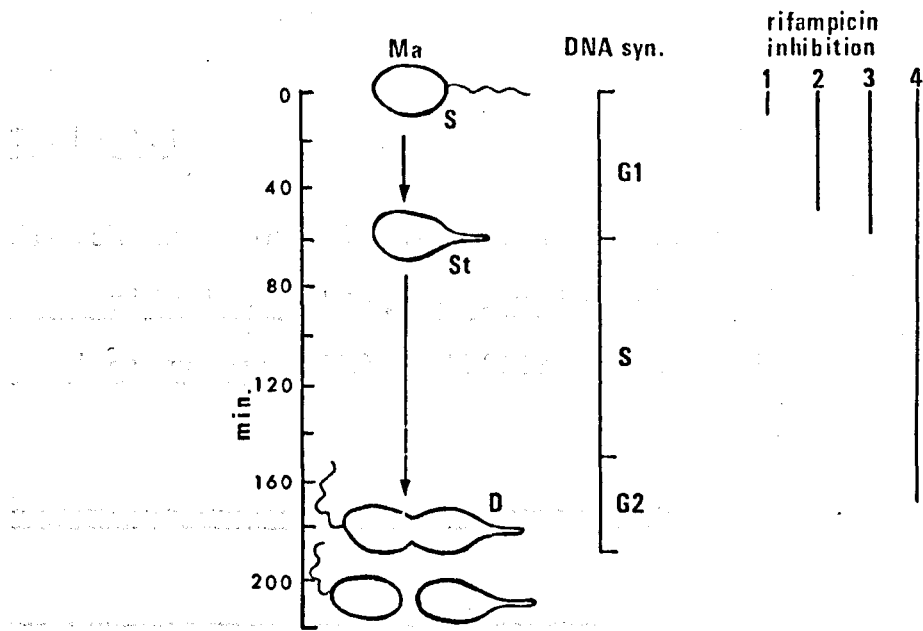


Figure 4.6

The cell cycle of Caulobacter crescentus (Newton, 1972)

Ma Morphological appearance.

S Swarm cell.

St Stalked cell.

D Dividing cell.

1. Loss of motility.

2. Stalk formation.

3. DNA synthesis initiation.

4. Cell division.

Table 4.1

Growth and morphological characteristics of
Rh. acidophila (RA), Rh. palustris (RP), and
Rhodomicrobium (RM) - differential gradient.

	RA	RP	RM
Obligate life cycle (Swarm cell → Mother cell)	+	+	+
Polar growth	+	++	+++
Asymmetric division	+	++	+++
Lamellar membrane system	+	+	+
Loss of motility	+	+	+
Maturation of swarm cell	+	+	+
Filament/tube formation	-	+	++
Branch/chain formation	-	-	++
Exospore formation	-	-	+

as a differential model system was investigated (concentrating on Rhodomicrobium) with a view to their exploitation as an alternative and more versatile synchronous differential system than those described in the literature.

Section 4.2Materials and Methods.a) Media.

Medium PYR (section 3.2, page 87.) was used for all experiments involving Rhodomicrobium.

Medium Rp contained per litre distilled water:

Sodium acetate	1.5g
Sodium pyruvate	1.5g
Yeast extract	1 g
Mineral salts as per PYR medium.	

Caulobacter was grown on G + Y medium (section 2.2, page 23) and Hyphomicrobium on All medium (section 2.2, page 23).

b) Organisms.

Caulobacter

Hyphomicrobium

Rhodopseudomonas palustris

Rhodomicrobium (Rm5).

The organisms used in this study have been described in previous sections.

c) Growth conditions for synchronised cells.

Photosynthetic cells were grown either in 1cm path-length, 3 ml stoppered glass cuvettes completely filled with medium so ensuring an anaerobic environment or in flasks of liquid medium as previously described (section 3.2, page 90). All cultures were incubated in thermostatically controlled, shaking waterbaths and illumination was by a 150 W tungsten light source giving a light intensity of 6-7,000 lux. Samples were taken for analysis by sterile,

disposable syringes.

Caulobacter and Hyphomicrobium were grown as previously described (section 2.2, page 22).

d) Large scale batch culture growth of Rhodomicrobium.

10 litre batch cultures of Rhodomicrobium (Rm5) were grown on PYR medium in an L.H. Engineering batch fermenter, fitted with temperature and pH control (plate 4.1). An aerobiosis was maintained by flushing with a continuous flow of oxygen free nitrogen (50 ml/min) and light energy was supplied by a 150W tungsten lamp (6,000 lux). Loss of light and culture shading was minimised by covering the enclosed three corners of the fermenter housing with reflective foil. Incubation was at 30°C with a stirring rate of 1,5000 rev/min. The pH was controlled when necessary by the addition of N HCl.

e) Selective cell synchronisation.

Separation of motile swarm cells from heterogenous batch culture of Rhodomicrobium (Rm5), Caulobacter and Rh. palustris was by filtration through a glass column containing alternate layers of glass wool and 80mesh glass chromatography beads, introduced to prevent streaming down the sides of the column.

The apparatus was of Quickfit glass ware designed and assembled to ensure sterility and definition of the environment throughout the filtration procedure (figure 4.7). Sterilisation was at 160°C overnight in a dry heat oven.

Heterogeneous cultures were introduced into the column under nitrogen at a flow rate of 30-40 ml/min; the first 50 ml was discarded and the subsequent filtrate was used as the synchronised population.

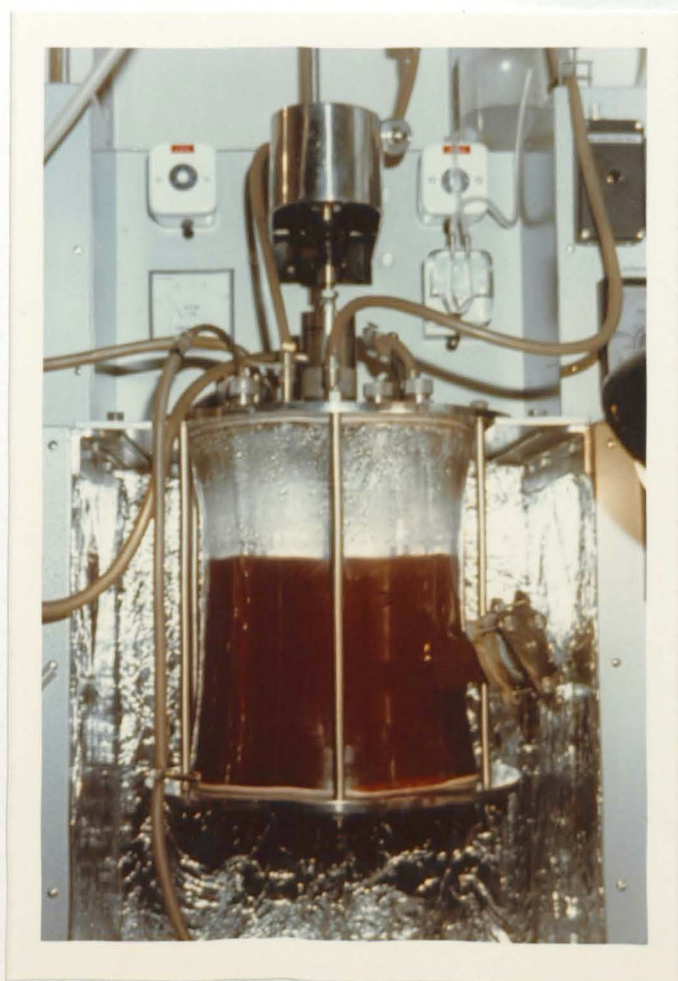
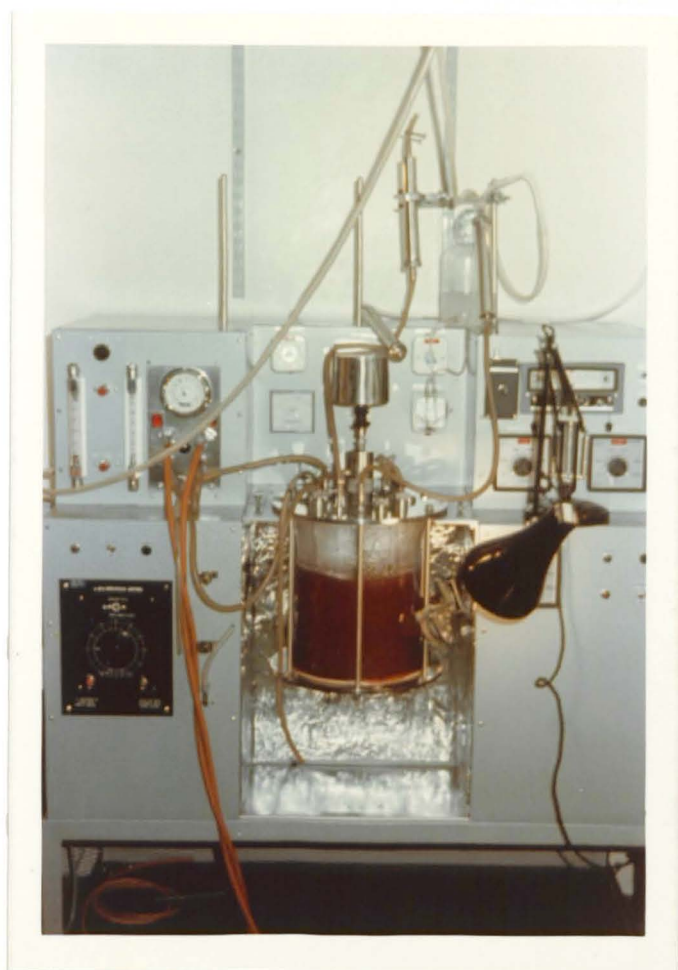


Plate 4.1 Batch culture of *Rhodospirillum rubrum*.

Figure 4.7.

Apparatus used for selective cell synchronisation of Rhodomicrobium.

gw Glass wool

gb 80 mesh glass chromatography
beads.

500 ml dropping funnel (D2)

Socket adapters (MF18)

Glass condenser (C2)

Erlenmeyer flask (FE250)

f) Measurement of synchronised cell growth.

Cell growth was followed by:

- i) spectrophotometry (section 3.2, page 94)
- ii) protein determination (Lowry et al, 1951)
- iii) dry weight determination.
- iv) viable count for Caulobacter and Hyphomicrobium.
- v) slide culture (section 2.2, page 27)
- vi) radioisotope incorporation.

Dry weight determination.

Dry weights were determined in acid clean glass centrifuge tubes which had been accurately weighed prior to culture addition. 5 ml samples were added, centrifuged and washed twice with distilled water prior to heating at 160°C in a dry heat oven to a constant weight.

Radioisotope incorporation.

Determination of the incorporation of exogenous thymine, thymidine, adenine, deoxyadenosine and deoxyguanosine into DNA was carried out by incubating cells in the presence of such material labelled with tritium (^3H).

Alternatively nucleic acid synthesis was followed by the uptake of phosphorus-32. DNA synthesis was determined by hydrolysing the samples with $\bar{\text{N}}$ NaOH for 30 minutes at 37°C followed by neutralisation with HCl and further incubation for 1 hr at 37°C in the presence of 100 $\mu\text{g/ml}$ ribonuclease so ensuring removal of RNA. Subtraction of the DNA determinations from those of the total nucleic acid indirectly quantified RNA synthesis.

The timed addition of the labelled material was facilitated by having a side arm attached to the flask. This solved the problem of the time lag between inoculating,

flushing (with oxygen free nitrogen), and incubation of the flask. At the required time the isotope, isolated in the side arm, was added to the culture medium.

Incorporation was stopped by precipitation with cold 10% w/v trichloroacetic acid (TCA) in the presence of 100 µg/ml of bovine serum albumin or cold carrier. Where necessary RNA was degraded by centrifuging the sample, resuspending in 1N KOH, incubating for 15 hrs. at 37°C, neutralising with 12N HCl and re-precipitating with 10% w/v TCA containing 100 µg/ml of the appropriate carrier. After a minimum of 30 minutes at 4°C samples were filtered on 2.5cm Whatman GF/C glass fibre filters, washed three times with 10% w/v TCA, ethanol and finally ether and dried at 105°C for 1 hr.

RNA, protein and chlorophyll synthesis were followed by measuring the uptake of (5-³H) Uridine, L-(4,5-³H)Leucine and 5 Amino(G-³H) laevulinic acid respectively.

Where incorporation of label into chlorophyll was being determined the solvent washes were excluded and the filters were dried overnight at 80°C.

Glass fibre filters were counted by immersion in scintillation fluid consisting of 36g 2-5-diphenyloxazole, plus 0.45g 1,4-bis-(5-phenyloxazole-2-yl) benzene dissolved in 6 litres of Toluene plus 3 litres of Triton X-100 on a Packard 3000 Series Tri-Carb liquid scintillation spectrometer.

g) DNA determinations.

DNA was determined fluoremetrically by the method of Donkersloot et al (1972) which is based upon the marked increase in fluorescence occurring when DNA is complexed

with ethidium bromide (LePecq and Paoletti, 1967). The fluorescence can be used as a measure of DNA and RNA concentrations either alone or in combination.

Donkersloot et al (1972) exhaustively verified the experimental procedure in a microbiological context showing both the sensitivity, versatility and the limitations of the system. However, to surmount the problem of background interference, necessary to achieve maximum sensitivity, and maximum changes in fluorescence it was found to be essential to equilibrate both DNA and ethidium bromide concentrations to a 1:1 ratio (figure 4.8) in addition to working within the range 0.2-1 μg DNA concentration i.e. linear relationship.

The correlation between growth rate and DNA concentration was verified using a Klebsiella species as a control microbiological system (figure 4.9).

As shown by Donkersloot et al (1972) there is no requirement to remove cellular debris as interference from such is negligible.

h) Inhibition of DNA synthesis.

Nalidixic acid (50 $\mu\text{g}/\text{ml}$) and Mitomycin c (5 $\mu\text{g}/\text{ml}$) were used to inhibit DNA synthesis in synchronously growing Rhodomicrobium swarm cell populations. The effects of the antibiotics were determined spectrophotometrically and microscopically by slide culture.

i) Giemsa stain.

Microbial samples were dropped onto acid cleaned microscope slides, dried in air and fixed in methanol for 10 minutes. Interfering RNA was hydrolysed by heating for 5 minutes at 60°C in the presence of N HCl followed by 1 hr.

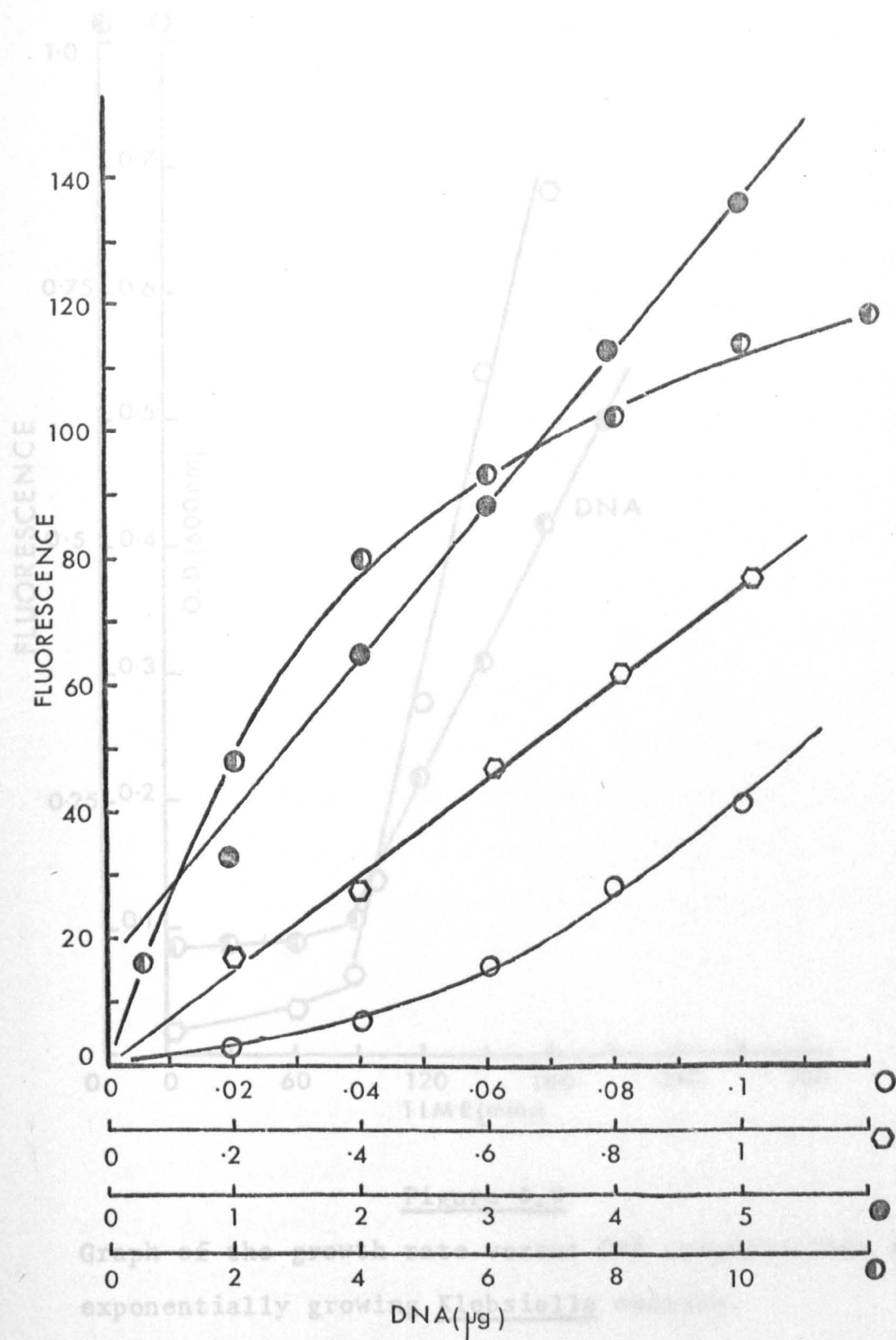


Figure 4.8

Graph of the relationship between DNA concentration and fluorescence with an ethidium bromide concentration of 1 μg/ml.

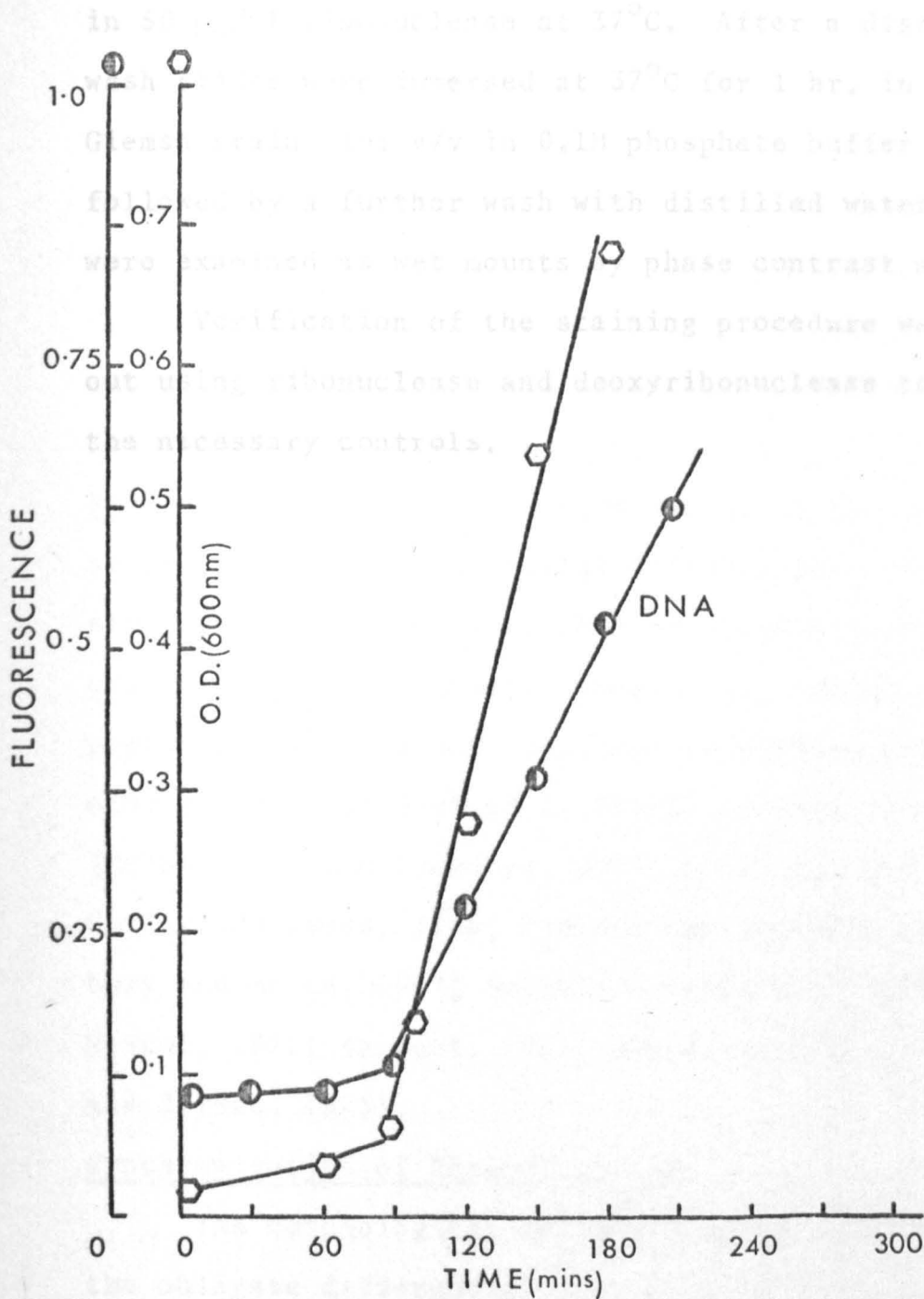


Figure 4.9

Graph of the growth rate versus DNA concentration of an exponentially growing Klebsiella culture.

in 50 µg/ml ribonuclease at 37°C. After a distilled water wash slides were immersed at 37°C for 1 hr. in dilute Giemsa stain (10% v/v in 0.1M phosphate buffer pH 6.8) followed by a further wash with distilled water. Slides were examined as wet mounts by phase contrast microscopy.

Verification of the staining procedure was carried out using ribonuclease and deoxyribonuclease to produce the necessary controls.

Section 4.3 Results and Discussion.

The central problem of differentiation relates to those endogenous mechanisms that make available in daughter cells genetic information that was not readily available in the mother cell. As discussed in the introduction several procaryotic organisms present such differential systems which are amenable to study in this context, eg. Caulobacter, Hyphomicrobium, Rh. acidophila, Rh. palustris, Rhodomicrobium. All reproduce following completion of a series of obligate sequential differential events. The study and exploitation of such an organised sequence of events, however, requires homogeneous, synchronised cell populations. This has long been recognised and many techniques have been evolved to obtain synchronised populations (Helmstetter and Cummings, 1963; Mitchison and Vincent, 1965; Cutler and Evans, 1966; Schmidt and Stanier, 1966; Whittenbury and McLee, 1967; Smith and Pardee, 1970; Degnen and Newton, 1972; Sargent, 1973; Moore and Hirsch, 1973; Staley and Jordan, 1973).

Synchronization of Rhodomicrobium.

The morphological and physiological diversity, and the obligate differential growth cycle of Rhodomicrobium (section 3.3) makes this genus particularly valuable as a model differentiation system. As with other differential models, eg. Caulobacter (Shapiro et al, 1971; Degnen and Newton, 1972) and Hyphomicrobium (Moore and Hirsch, 1973) the initial problem is to obtain quantities of selectively synchronised populations by the simplest non-physiological technique.

The method developed to achieve synchronisation of

Rhodomicrobium exploits the two morphological extremes of this organism found in middle and late exponential photosynthetic cultures i.e. colonial chain forming mother cells and motile swarm cells. In essence, synchronised swarm cell populations are obtained by passing a heterogeneous exponential culture through a glass wool:Ballotini bead column under strictly controlled environmental conditions (section 4.2, page 238). The integral colonial morphology of the mother cells results in them being retained on the column; the motile swarm cells pass through unhindered. Unlike the somewhat comparable technique used to synchronise Caulobacter (Staley and Jordan, 1973)

Rhodomicrobium is not grown within the confines of the column; this serves only as an environmentally defined filtration system. In contrast to the Caulobacter methods which are dependent primarily on adhesion by the holdfast of the 'mother' cell to glass surfaces (the physiology and extent of which is unknown and likely to be inconsistent) synchronisation of Rhodomicrobium exploits the extensive morphological differences between two extreme cell types.

To prevent any physiological shock, particularly exposure of the anaerobically grown cells to oxygen, the column is flushed with oxygen-free nitrogen and maintained at the culture incubation temperature and light intensity, i.e. there is no alteration of the cellular environment during the synchronisation process and in consequence there is likely to be no disruption or alteration of the cellular metabolism.

The culture volume of the synchronised Rhodomicrobium cell population is limited only by the rate of blockage of the column by the retained mother cells. The technique

described (section 4.2, page 237) yields 250-300 mls of a cell population of $1-2.5 \times 10^7$ cells/ml (approximately) which is comprised of 97-98% of swarm cells (as determined microscopically)(plate 4.2). The time factor involved in synchronisation is approximately ten minutes.

The problem of blockage and in consequence limitation of the synchronised culture volume obtained from the column may be overcome by a simple modification which facilitates passage of very large heterogeneous culture volumes. This involves a glass wool pre-filter of large surface area, approximately two to three inches in depth which retains the majority of chain formations prior to passage into the synchronisation column.

The number of viable cells/ml of the synchronised population is dependent solely on the physiological state of the pre-filtered heterogeneous culture. This can however, be grown consistently to a known cellular content by accurately monitoring the variable parameters eg. pH, temperature, light intensity and oxygen concentration in a batch culture fermenter (section 4.2, page 235).

In contrast to this method, the method of Degnen and Newton (1973), performed in a glass petri dish containing a stirred heterogeneous Caulobacter population (dependent on adhesion of the stalked hofast cells to the glass perimeter) yields 5-8 mls of a suspension containing $1-3 \times 10^7$ viable cells/ml. That of Staley and Jordan (1973), scaled up to the extent of culturing the Caulobacter cells in a 500 ml separating funnel containing 4 mm glass beads (large surface area), yields a maximum of 150 ml having a population density of 5.9×10^6 cells/ml. The centrifugation-filtration

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Rhodospirillum rubrum
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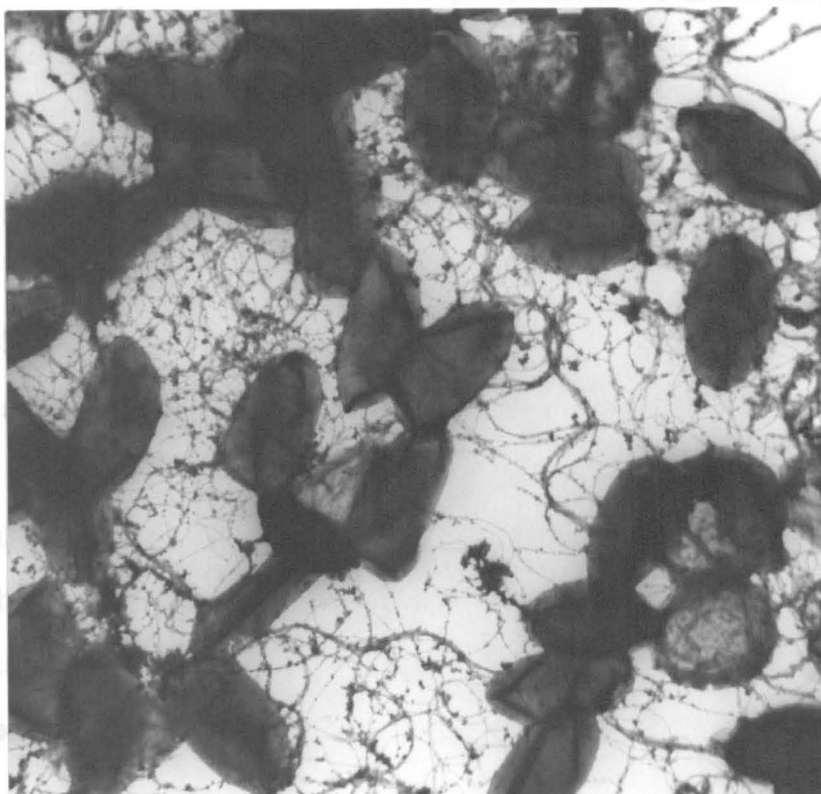
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Synchronisation of other Plate 4.2

Synchronised Rhodospirillum rubrum swarm cell population
Rhodospirillum rubrum as prepared by the column technique. Uranyl acetate
 plotted (0.5% w/v) negative stain.

from stationary phase cultures. The synchronised populations of Rhodospirillum rubrum obtained simply by passing a stationary phase culture through a column under appropriate environmental conditions. The column at a flow rate of 10 ml/min. The synchronisation described methods synchronisation of Rhodospirillum rubrum is dependent on adhesion to the glass fibres. The synchronisation fast of the 'mother' stalled cells. The synchronisation procedure additionally retains the synchronised cells by trapping them within the glass fibres. Figure 4.2 illustrates the

synchronisation procedure of Moore and Hirsch (1973) for Hypomicrobium gave 30 mls of a culture containing 2×10^7 cells /ml.

Table 4.2 summerises the details of the synchronisation procedures employed in the above differentiation systems.

In summation the above synchronisation technique allows separation of one distinct cell type (the swarmer cell) from all other morphological cell types of Rhodomicrobium. The quantitative aspects bear little comparison with techniques employed in other procaryotic differential systems. There is no discernable physiological stress imposed on the culture since the environment can be strictly defined. The technique is simple, hence constant, and reliable having no dependence on the physiological characteristics of the cells.

Synchronisation of other appendaged, dimorphic procaryotes.

Although designed primarily for synchronisation of Rhodomicrobium swarm cells the above technique can be exploited to obtain populations of Rhodomicrobium exospores from stationary phase cultures. In addition, however, synchronised populations of Caulobacter swarm cells may be obtained simply by passing a heterogeneous stalked population, under appropriate environmental conditions, through the column at a flow rate of 10 mls/min. As with the previously described methods synchronisation of Caulobacter swarm cells is dependant on adhesion to the glass surface by the hold-fast of the 'mother' stalked cell. However, the column procedure additionally retains the stalked cells by trapping them within the glass fibres. Figure 4.10 illustrates the

Table 4.2

Organism	Synchronisation procedure.	Vol. of culture(ml)	No. cells/ml	rf.
<u>Caulobacter</u>	i) filtration	not given		1
	ii) density gradient sedimentation.	2-3	not given	2
	iii) adhesion of the mother cell.	3-4	$1-3 \times 10^7$	3
	iv) " "	150	5.9×10^6	4
<u>Hyphomicrobium</u>	centrifugation, filtration.	30	2×10^7	5
<u>Rh. palustris</u>	density gradient sedimentation	2-3	1.4×10^9	6
<u>Rhodomicrobium</u>	filtration	250-300	$1-2.5 \times 10^7$	7

References.

1. Schmidt and Stanier (1966)
2. Shapiro (1971)
3. Degnen and Newton (1972)
4. Staley and Jordan (1973)
5. Moore and Hirsch (1973)
6. Whittenbury and Westmacot (1973)
7. This thesis.

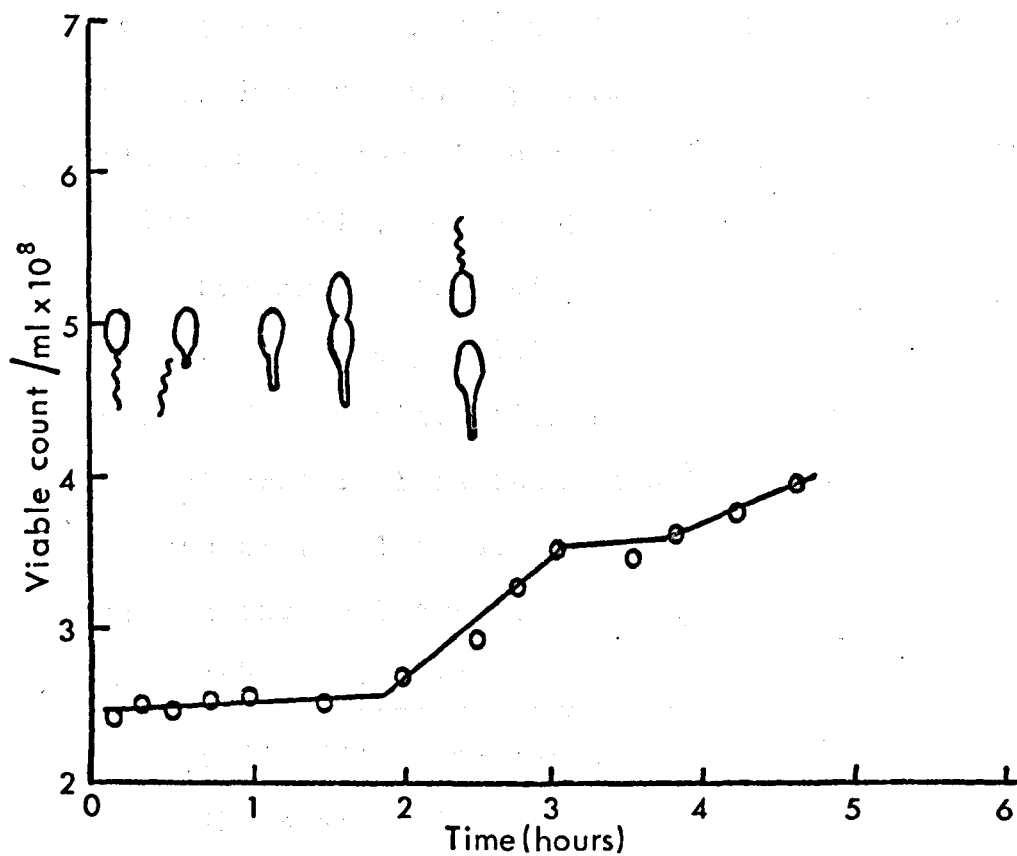


Figure 4.10

One step growth curve obtained by incubating a column synchronised Caulobacter swarm cell population.
(Morphology of cell type as observed microscopically)

one step growth curve obtained on incubating a Caulobacter swarm cell population obtained by column synchronisation. The cell yield/ml is comparable with those reported in the literature for other procedures. The column technique however involves no physiological stress, yields considerable volumes of synchronised cells, is rapid and well defined environmentally, hence physiologically.

Synchronisation of Hyphomicrobium and Rh. palustris cultures is also possible by the above procedure but the degree of synchronisation (percentage of one cell type in the culture) is less than with Rhodomicrobium, presumably because of the less striking morphological differences between the cell types.

The morphological and physiological aspect of Rhodomicrobium swarm cell differentiation.

The swarm cell cycle of Rhodomicrobium was selected for study because it displays an obligate, temporal sequence of differentiation events which are amenable to study at the molecular level i.e. a model differentiation system. (All synchronous growth experiments were performed on photosynthetically grown cultures i.e. light intensity of 6,000 lux with incubation at 30°C under strictly anaerobic conditions)

i) Morphological aspects of the differentiation cycle.

The obligate, temporal morphological sequences expressed during the Rhodomicrobium swarm cell cycle have been analysed both by microscopic slide culture (section 3.3, page 146) and as synchronised swarm cell populations. Figure 4.11 illustrates diagrammatically the temporal sequence of events as determined by following synchronised populations in liquid (photosynthetic) culture by both light and electron microscopy.

MorphologyTime (mins.)

1.Highly motile

0-20



2.Loss of motility

20-30



3.Swarm cell maturation

30-150



4.Filament synthesis

150-210



5.Bud formation

210-390

6.Completion of
daughter cell

420



7.Filament synthesis.

Figure 4.11

Sequential morphological events occurring during differentiation of the Rhodomicrobium swarm cell.

To follow synchrony of Rhodomicrobium swarm cells by viable or particle counts/ml is not possible since upon daughter cell maturation both cells, although physiologically isolated, remain attached. A characteristic which isolates this model from the other procaryotic differentiation systems eg. Caulobacter (Shapiro et al, 1971) Hyphomicrobium (Moore and Hirsch, 1973) and Rh. palustris (Whittenbury and McLee, 1967). However, the growth cycle can be followed by optical density readings at 540nm (not affected by photopigment absorption) in conjunction with microscopic observations as illustrated in figure 4.12. The obligate temporal nature of growth accompanied by distinct morphological changes allows the point reached in the differentiation sequence to be determined simply by microscopic examination i.e. synchronous growth is characterised by a one step growth curve which may be subdivided and associated with specific and exclusive morphological forms (figure 4.12).

There is no lag period expressed by the synchronous swarm cell cultures showing that the cells have not been subjected to adverse physiological stresses. The most critical environmental parameter which adversely affects the growth of these cells (presence of oxygen) is controlled by the maintenance of strictly anaerobic conditions. Oxygen concentrations were monitored chromatographically throughout these experiments (section 3.2, page 91).

ii) Physiological aspects of the differentiation cycle.

a) Protein synthesis.

Protein synthesis during the Rhodomicrobium swarm cell cycle, as determined by absorption at 540nm, has been

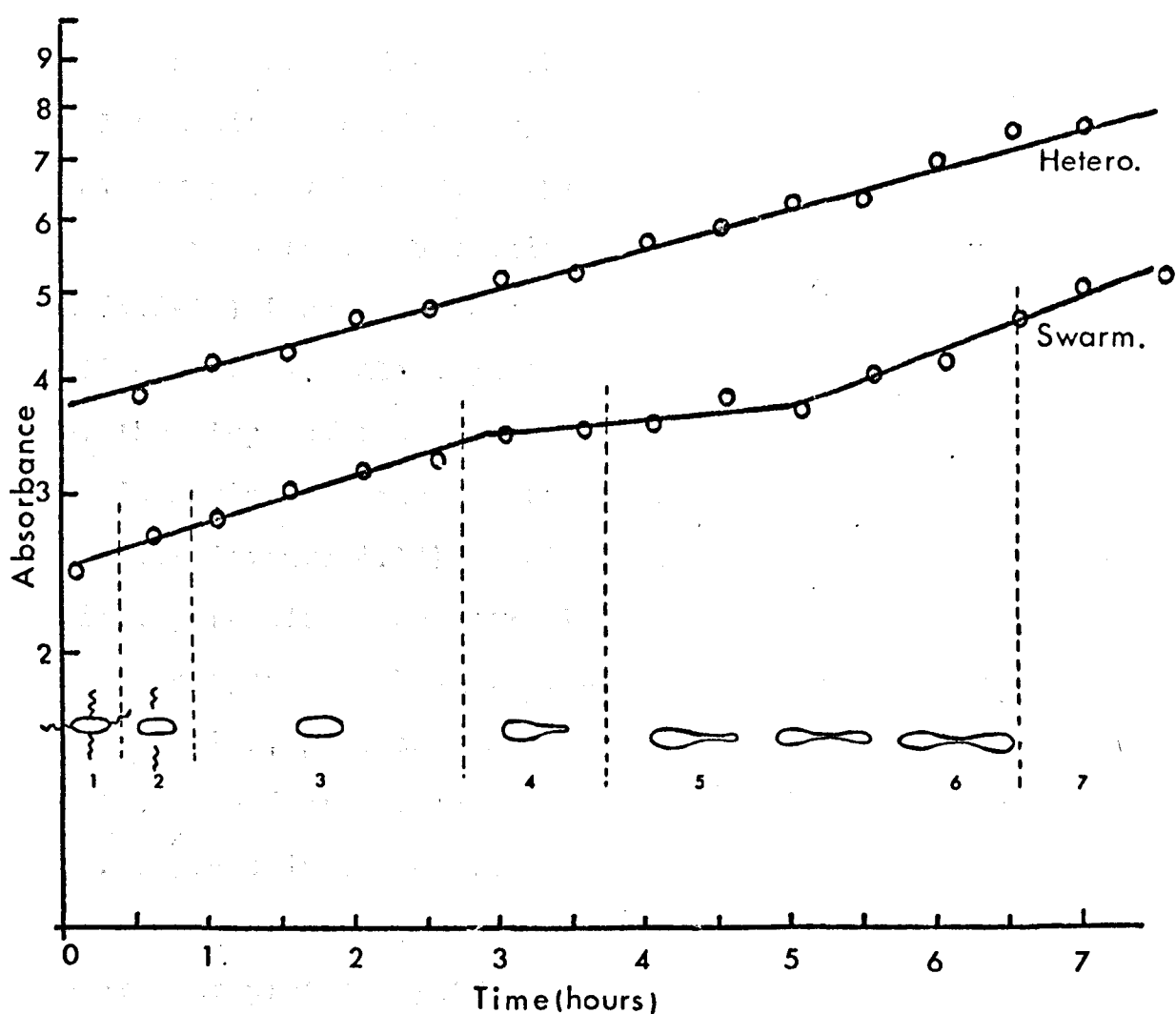


Figure 4.12

Growth of a synchronised Rhodospirillum rubrum swarm cell population (Swarm) in comparison with growth of a heterogeneous culture (Hetero). Growth measured by absorption at 540nm. Subdivision of the synchronous cycle is based on light and electron microscopic observations.

shown as a stepped curve the slopes of which can be correlated with specific morphological events (figure 4.12). This graph, with relation primarily to protein synthesis, has been further substantiated by following the uptake of L-(4,5- ^3H) Leucine by a synchronous swarm cell population (figure 4.13). Although there is a break in the continuity of the slope of the curve for the synchronised population, it is not as distinct as those produced from absorption studies (figure 4.14). This is not necessarily a contradictory result since it is logical to predict that in a dynamic system such as that presented by differentiating swarm cells, protein synthesis must approach a constant rate. The decrease in rate of incorporation of ^3H leucine corresponds in the differential cycle to initiation of filament formation, a period when the quantitative requirements for protein synthesis may be envisaged as decreasing since the maturation process is complete (further indirect evidence of this will be presented on considering chlorophyll synthesis below).

b) Chlorophyll synthesis.

Absorption measurements taken at 875nm throughout a synchronous growth cycle gave a graph indistinguishable from that for absorption at 540nm (figure 4.14). To ensure specificity of the chlorophyll determination (synthesis) the uptake of 5-amino(G- ^3H)laevulinic acid was monitored.

(5-amino laevulinic acid is a common intermediate in the biosynthesis of vitamin B₁₂, haems and chlorophyll, and if formed from glycine and succinyl CoA by the action of 5-amino laevulinic acid synthetase)(Lascelles and Altshuler, 1969; Fanica-Gaignier and Clement-Metral, 1971). The

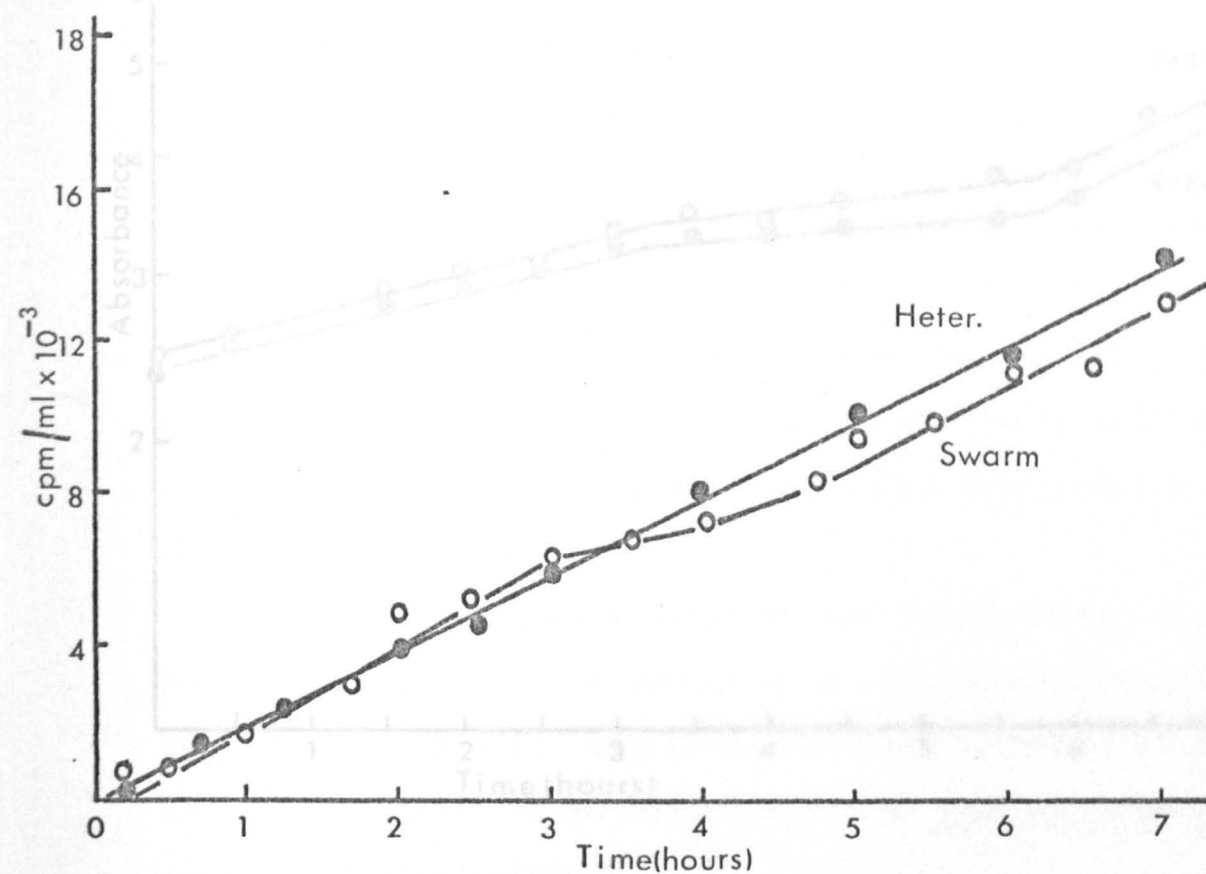


Figure 4.13

Absorption curves at Figure 4.13

Uptake of L-(4-5-³H) leucine by synchronised (Swarm) and heterogeneous (Hetero.) cultures of Rhodomicrobium.

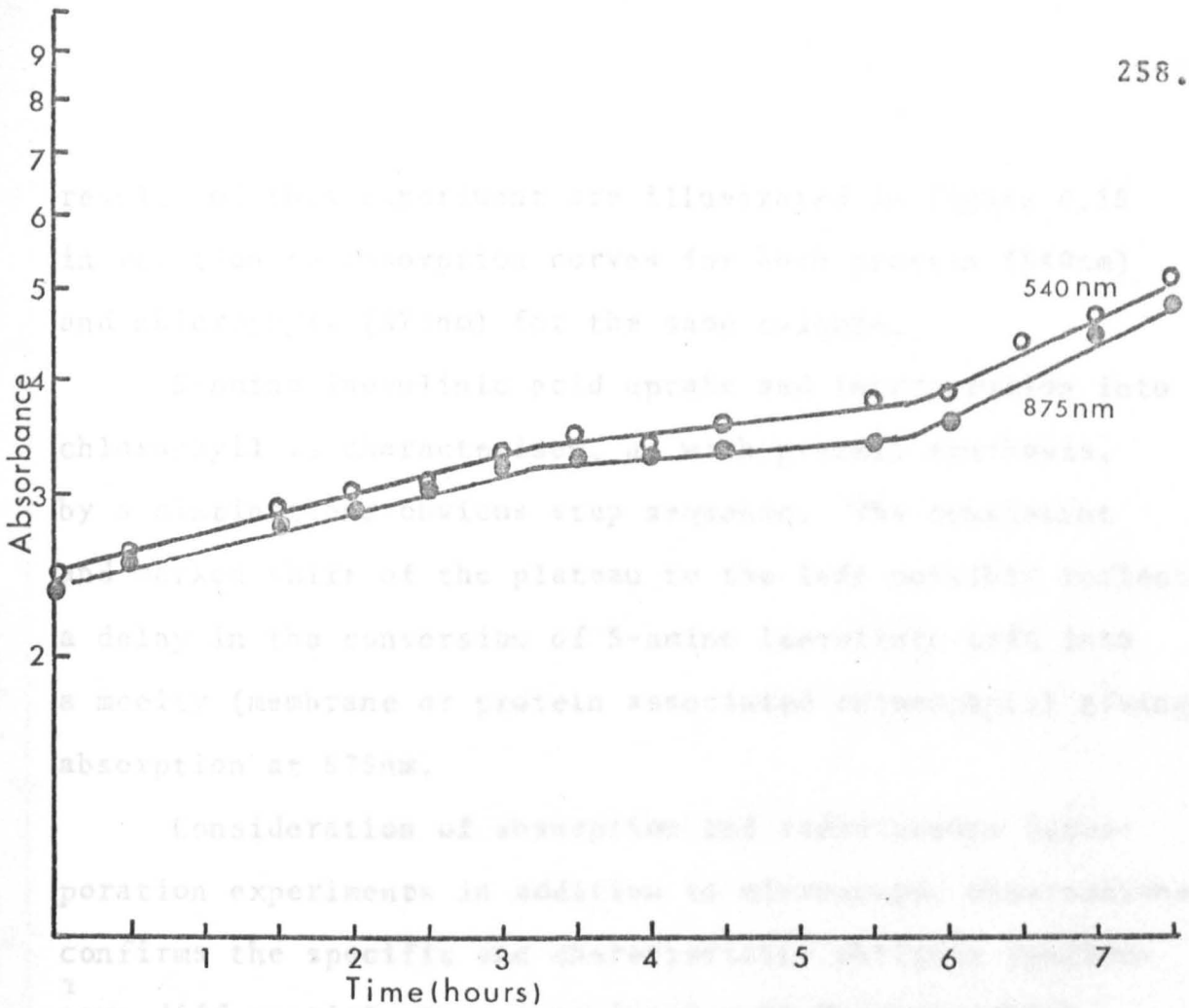


Figure 4.14

Absorption curves at 540nm (O) and 875nm (●) for synchronous growth of Rhodomicrobium swarm cells.

results of this experiment are illustrated in figure 4.15 in relation to absorption curves for both protein (540nm) and chlorophyll (875nm) for the same culture.

5-amino laevulinic acid uptake and incorporation into chlorophyll is characterised, as with protein synthesis, by a distinct and obvious step sequence. The consistent and marked shift of the plateau to the left possibly reflects a delay in the conversion of 5-amino laevulinic acid into a moiety (membrane or protein associated chlorophyll) giving absorption at 875nm.

Consideration of absorption and radioisotope incorporation experiments in addition to microscopic observations confirms the specific and characteristic obligate synchronous differential steps associated with Rhodomicrobium swarm cell development.

During the initial period of growth undergone by developing swarm cells i.e. 0-180 minutes, there is intense physiological (metabolic) activity. Since this occurs prior to filament synthesis and there is no obvious morphological alteration there must be considerable internal (ultrastructural) synthesis; this can be explained as de novo synthesis of the chlorophyll containing lamellar membrane system within the swarm cell. Protein and chlorophyll growth determinations support this idea. Additionally, until the cell has synthesised an efficient energy yielding system cell growth and reproduction is not possible. Therefore the initial metabolic activity can be summarised as maturation of the swarm cell i.e. a reflection of the synthesis of the lamellar membrane system. Considering the growth curve further, the plateau represents filament

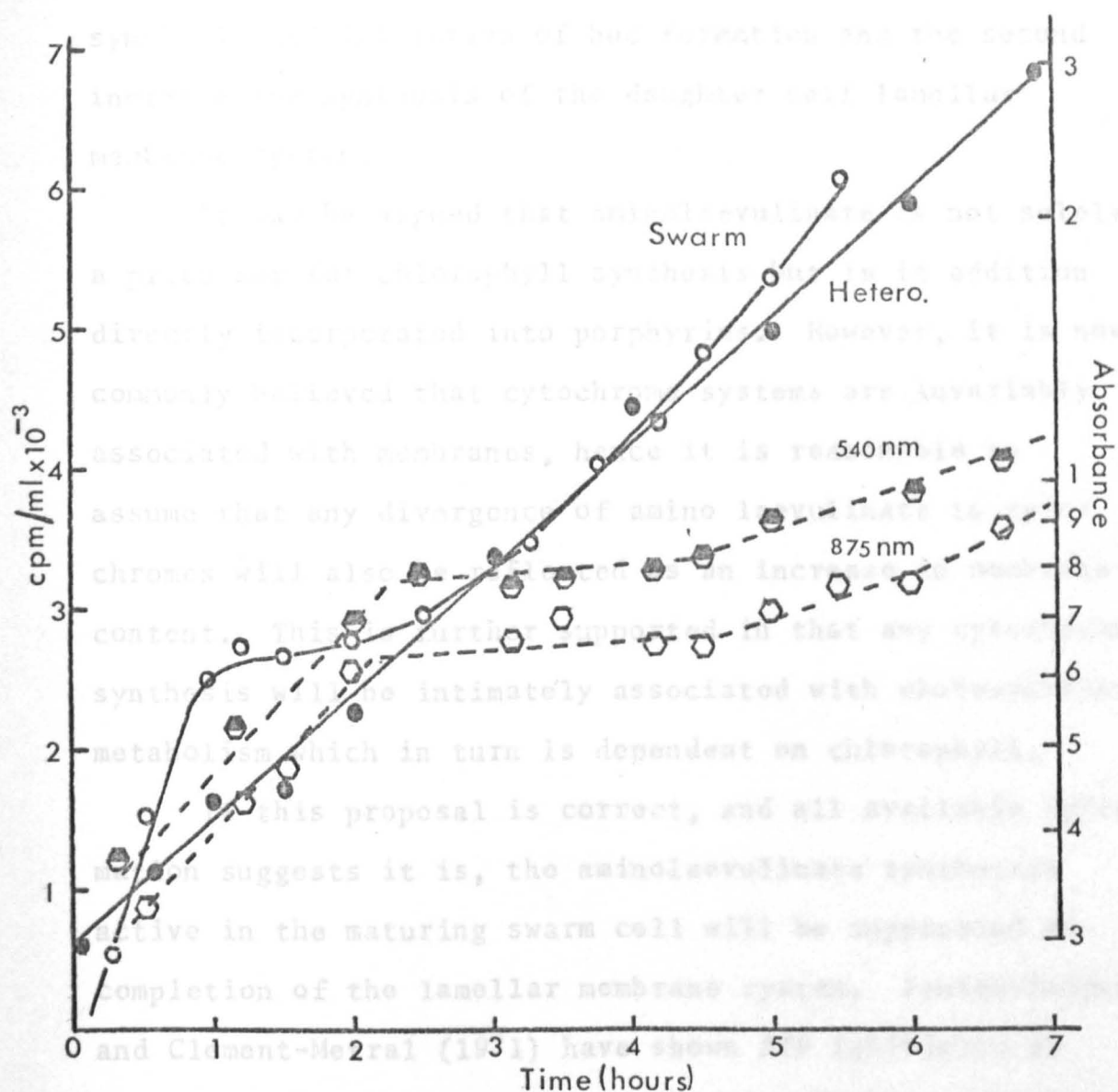


Figure 4.15

Uptake of ^3H 5-aminolaevulinic acid by heterogeneous (Hetero. ●) and synchronous (Swarm ○) Rhodospirillum rubrum cells in comparison to absorption curves at 540nm (●) and 875nm (○) for the same culture.

synthesis and initiation of bud formation and the second increase the synthesis of the daughter cell lamellar membrane system.

It may be argued that aminolaevulinate is not solely a precursor for chlorophyll synthesis but is in addition directly incorporated into porphyrins. However, it is now commonly believed that cytochrome systems are invariably associated with membranes, hence it is reasonable to assume that any divergence of amino laevulinate to cytochromes will also be reflected as an increase in membrane content. This is further supported in that any cytochrome synthesis will be intimately associated with photosynthetic metabolism which in turn is dependent on chlorophyll.

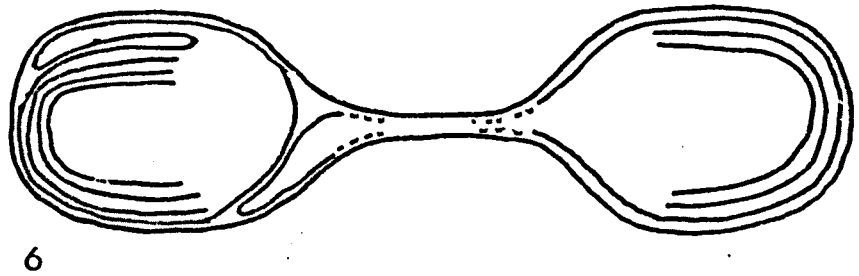
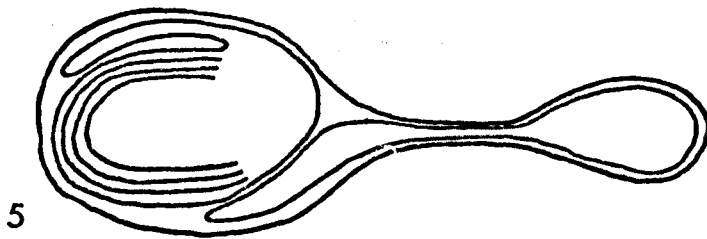
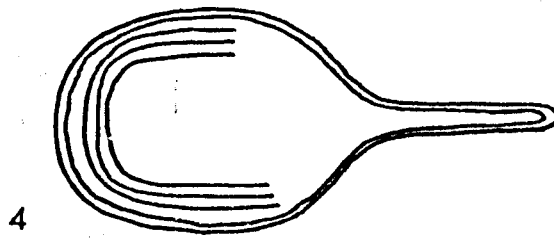
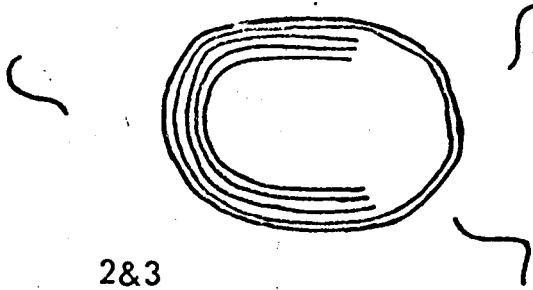
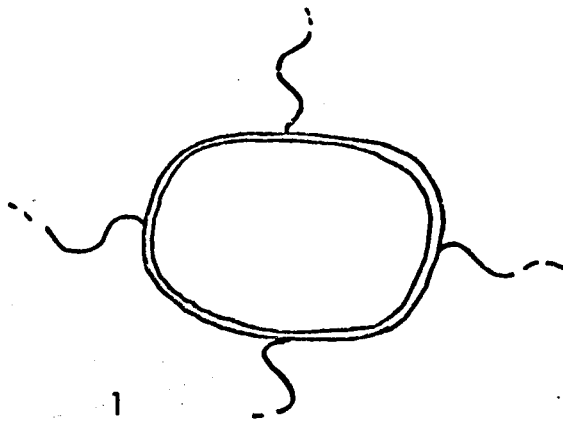
If this proposal is correct, and all available information suggests it is, the aminolaevulinate synthetase active in the maturing swarm cell will be suppressed on completion of the lamellar membrane system. Fanica-Gaignier and Clement-Metral (1971) have shown ATP inhibition of aminolaevulinate synthetase in Rhodopseudomonas sphroides. It is not unlikely therefore that a similar system operates in Rhodomicrobium. On completion of the lamellar membrane (photosynthetic) system ATP levels will rise and the amino-laevulinate synthetase will be inhibited. In contrast, in the developing daughter cell ATP levels will be appreciably lower, due to the lack of an energy yielding lamellar membrane complex, and aminolaevulinate synthetase will be induced. The important factor is that within a well-defined, obligate, sequential differentiation sequence there will be specific repression and induction of an enzyme whose activity can be monitored precisely within the cycle.

All of the above observations are, in addition, consistent with the "unit" cell growth hypothesis proposed in section 3.3 and summarised in relation to the synchronous growth cycle in figure 4.16.

1. Synchronous population of highly motile swarm cells. Little or no lamellar membrane system.
- 2.& 3. Loss of motility, extensive synthesis of the photosynthetic membrane system and initiation of synthesis of the daughter cell 'unit'.
4. Filament synthesis, ceasation of lamellar membrane synthesis i.e. inhibition of aminolaevulinate synthetase.
5. Bud formation, movement of daughter cell 'unit'.
6. Initiation of synthesis of daughter cell lamellar membrane complex i.e. induction of aminolaevulinate synthetase. Second daughter cell 'unit' formation.

Figure 4.16 A

Postulated ultrastructural details relating to the synchronous cycle.



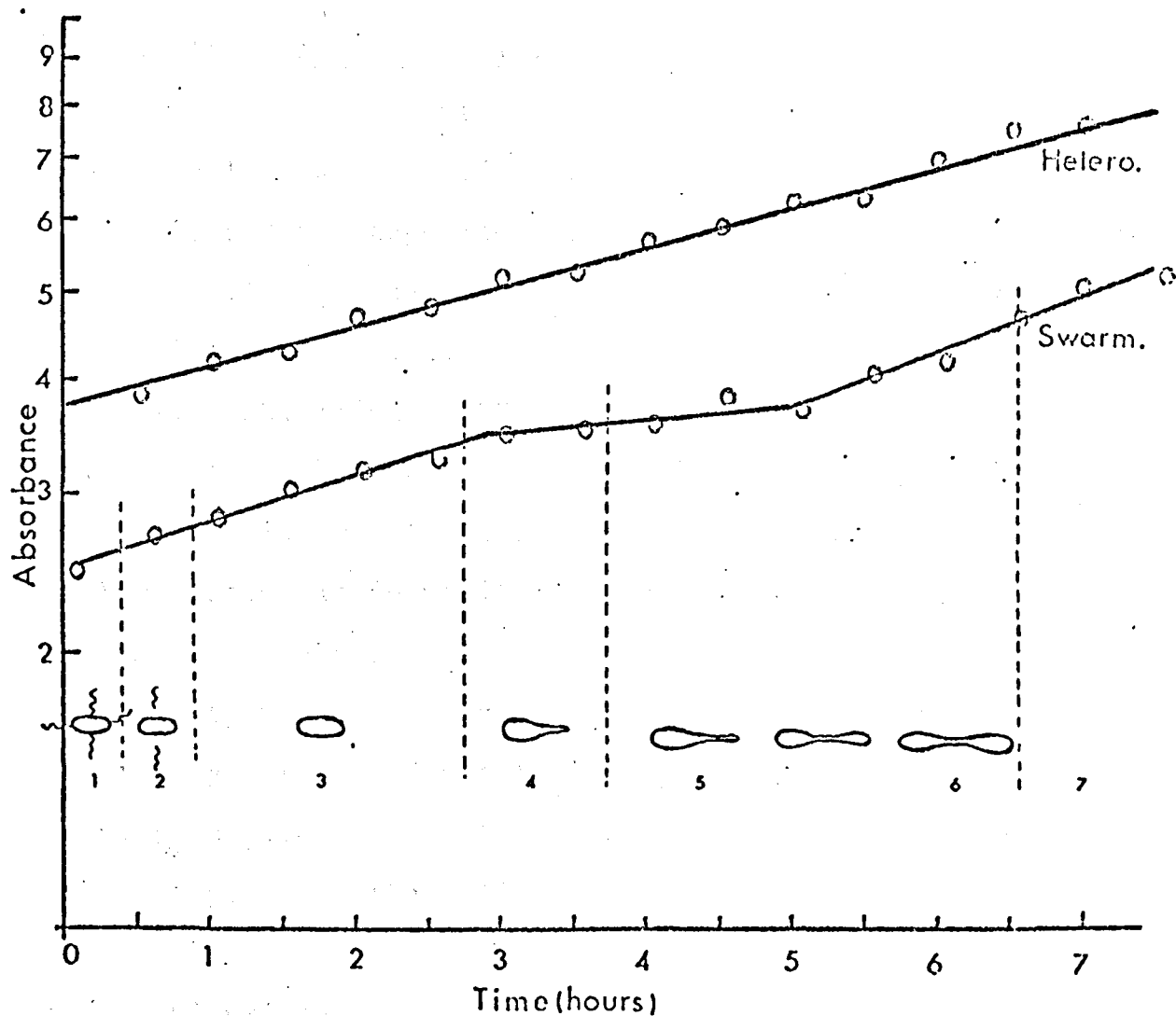


Figure 4.16 B

Growth of a synchronised Rhodomicrobium swarm cell population (Swarm) in comparison with growth of a heterogeneous culture (Hetero). Growth measured by absorption at 540nm. Subdivision of the synchronous cycle is based on light and electron microscopic observations.

c) Nucleic acid synthesis.

Nucleic acid synthesis in synchronised Rhodomicrobium swarm cells was examined in four ways:

- i) radioisotope incorporation.
- ii) fluorescence.
- iii) Giemsa stain.
- iv) inhibition of DNA synthesis.

i) The standard experimental procedure used to determine the synthesis or rate of synthesis of nucleic acid in cellular systems is to monitor the uptake and incorporation of radioactively labelled bases into the nucleic acids. Difficulty was encountered with Rhodomicrobium cells in that they were found not to incorporate any of the exogenously supplied radioactive bases into DNA or RNA with the exception of the following:

- a) Thymidine
- b) uridine.

a) Thymidine utilisation.

The pyrimidine of preference when studying DNA synthesis is obviously thymidine simply because it appears in DNA and only very exceptionally in RNA. Unfortunately uptake and incorporation of (methyl-³H) thymidine into the DNA of heterogeneously growing Rhodomicrobium cells was found to be low and insignificant. However, there is significant labelling of synchronous swarm cell DNA during the first hour of the growth cycle; thereafter it stops and label is effectively 'lost' (irrespective of the initial concentration of label present)(figure 4.17). The question therefore arises as to whether this is a physiological reflection of DNA synthesis within the synchronised population or simply an artifact. It is known that thymidine incorporation into DNA of microorganisms ceases after a short

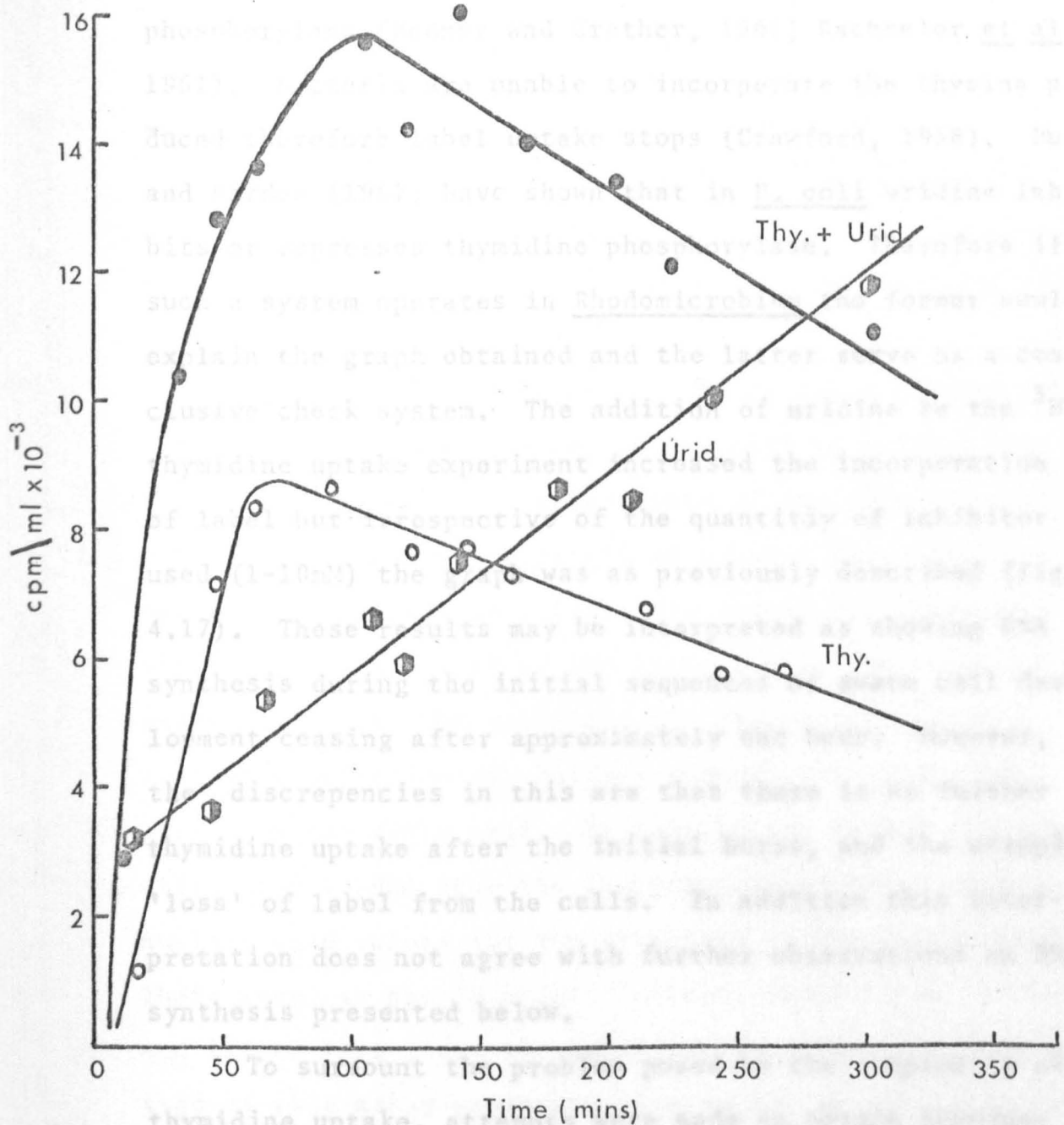


Figure 4.17

Uptake and incorporation by a synchronised Rhodomicrobium cell culture of (methyl- ^3H) thymidine (2 $\mu\text{Ci/ml}$ plus 2mM carrier) with (●) and without (○) uridine (2mM) being present. (⊙) The uptake and incorporation of ^3H -uridine (2 $\mu\text{Ci/ml}$ plus 2mM carrier) by a synchronous population. of RNA synthesis, was found, as far as the procedure allowed, to be linear throughout the period of

time due to degradation of thymidine to thymine by thymidine phosphorylase (Bodmer and Grether, 1965; Rachmeler et al, 1961). Bacteria are unable to incorporate the thymine produced therefore label uptake stops (Crawford, 1958). Budman and Pardee (1967) have shown that in E. coli uridine inhibits or represses thymidine phosphorylase. Therefore if such a system operates in Rhodomicrobium the former would explain the graph obtained and the latter serve as a conclusive check system. The addition of uridine to the ^3H thymidine uptake experiment increased the incorporation of label but irrespective of the quantity of inhibitor used (1-10mM) the graph was as previously described (figure 4.17). These results may be interpreted as showing DNA synthesis during the initial sequences of swarm cell development ceasing after approximately one hour. However, the discrepancies in this are that there is no further thymidine uptake after the initial burst, and the unexplained 'loss' of label from the cells. In addition this interpretation does not agree with further observations on DNA synthesis presented below.

To surmount the problem posed by the complexity of thymidine uptake, attempts were made to obtain thymine-requiring strains of Rhodomicrobium by mutation (N-methyl-N'-nitro-N-nitrosoguanidine, Ethyl methanesulfonate, Nitrous acid) and by trimethoprim selection (Stacey and Simson, 1965; Andrew, 1973). Neither were successful.

b) Uridine utilisation.

Uridine uptake and incorporation, a direct reflection of RNA synthesis, was found, as far as experimental procedure allowed, to be linear throughout the growth cycle of

a synchronised population i.e. there was no indication of fluctuation in the rate of synthesis during the cycle (figure 4.17).

ii) fluorometric determination of DNA synthesis.

An alternative assay system for DNA synthesis has been proposed by Donkersloot et al (1972) where the nucleic acid is complexed with ethidium bromide and the resulting fluorescence measured.

Synchronised Rhodocrobium swarm cells assayed for DNA synthesis by this procedure revealed an initial lag in synthesis followed by a steady rate of increase (figure 4.18). Heterogeneous populations treated in a similar manner did not show this lag. The former was therefore attributed to the lack of DNA synthesis in the immature swarm cell, i.e. DNA synthesis was discontinuous being initiated approximately halfway through the maturation period of the swarm cell. By subtraction of the fluorescence attributed to DNA from the fluorescence of total cellular nucleic acid values for the synthesis of RNA were obtained. These were, as per ^3H uridine uptake, found to be constant and linear throughout the synchronous cycle. The disadvantage of this assay procedure lies in the quantity of cellular material required for each fluorometric determination (5 mls).

iii) Giemsa stain.

A synchronously growing swarm cell population was sampled at regular intervals throughout the cycle and stained for DNA by Giemsa staining, after removal of interfering RNA by hydrolysis, (section 4.2, page 241) so allowing visualisation of the DNA in the light microscope (plate 4.3).

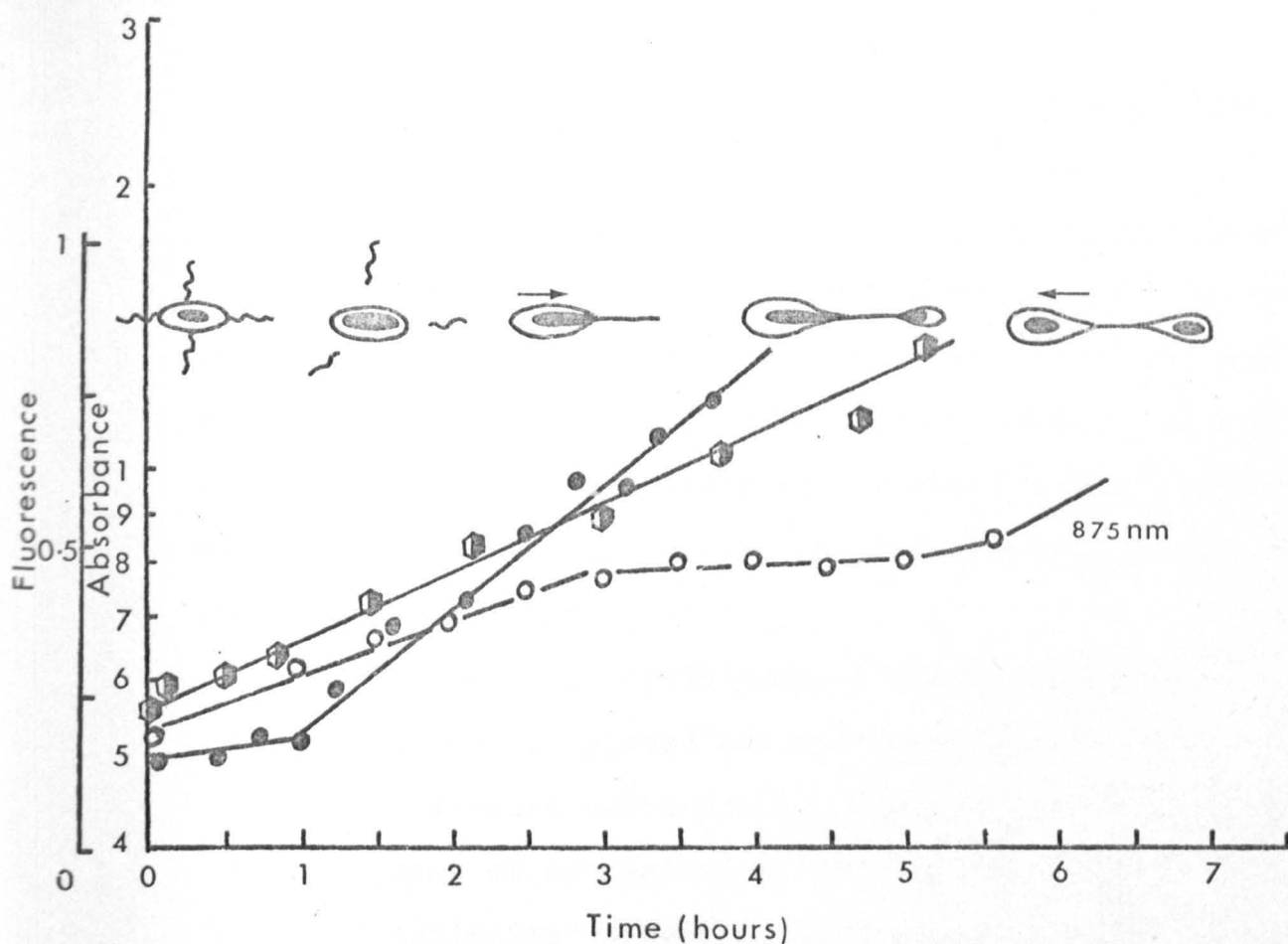


Figure 4.18

The relationship between DNA synthesis (●), the cytological appearance of the nuclear apparatus and the synchronous growth cycle of Rhodospirillum rubrum swarm cells (the one step growth curve illustrated as chlorophyll absorption at 875nm (○)).

The fluorometric determination of DNA synthesis in an exponentially growing heterogeneous culture (●) is shown in contrast to that of a synchronised population (●).

Plate 4.3

Electron micrographs of thin sections of Rhodomicrobium show the same diffuse fibrillar (DNA) structure typical of other bacterial nucleoids. Characteristic morphologies and specific movements were attributed to the cell 'nucleus' at specific points in the differential cycle. These are shown diagrammatically in figure 4.18 in relation to the synthesis of chlorophyll (absorption 875nm) and DNA (determined fluorometrically) during the synchronous growth cycle and were as follows:

- a) Motile swarm cells possess relatively small 'nucleoids' placed centrally within the cell.
- b) At a point corresponding approximately to the point of initiation of DNA synthesis the 'nucleoids' assume a characteristic ovoid shape and become much larger.
- c) Prior to filament formation there is movement of the ovoid, elongated structure to the pre-determined filament forming pole.
- d) From the time of bud formation DNA can be detected within the developing cell.
- e) When the developing daughter assumes approximately half the size of the mother cell the mother cell 'nucleoid' moves back to a central position.

The reproductive mechanism of Rhodomicrobium vannielii and the accompanying nuclear changes were studied by Murray and Douglas (1960) but only within the context of chain-forming cells. However, the observations they made are essentially similar to the above i.e. they observed 'bar-shaped' nuclear forms and in addition associated nuclear

material with the buds by the time they are first recognised as such. No nuclear material could be shown within the filaments.

Therefore macromolecular synthesis and the location and appearance of the nucleoid during the growth cycle indicate that DNA replication occurs during swarm cell maturation and is well established prior to filament synthesis.

iv) inhibition of DNA synthesis.

Nalidixic acid is an antibiotic which selectively inhibits DNA synthesis in Gram -ve bacteria (Goss et al, 1965). but the precise mode of action is not known (Boyle et al, 1969; Eberle and Masker, 1971; Puga and Tessman, 1973). However, the antibiotic has considerable value in studies linked to initiation and regulation of DNA replication within synchronised populations (Degnen and Newton, 1972; Weiner and Blackman, 1973) because it's effect can be reversed by dilution for a considerable period of time after addition.

Mitomycin c is another specific DNA inhibitor but in contrast to nalidixic acid its effect cannot be reversed.

Preliminary experiments were performed on synchronously growing Rhodomicrobium swarm cells using both inhibitors to give an indication as to whether continued development was coupled to DNA replication i.e. which morphogenetic stages of the cell cycle are dependent upon chromosome replications. Figure 4.19 illustrates the effect of nalidixic acid (50 µg/ml) and mitomycin c (5 µg/ml) on a synchronous Rhodomicrobium population. (Concentrations of both antibiotics used were well below bacteriocidal concentrations).

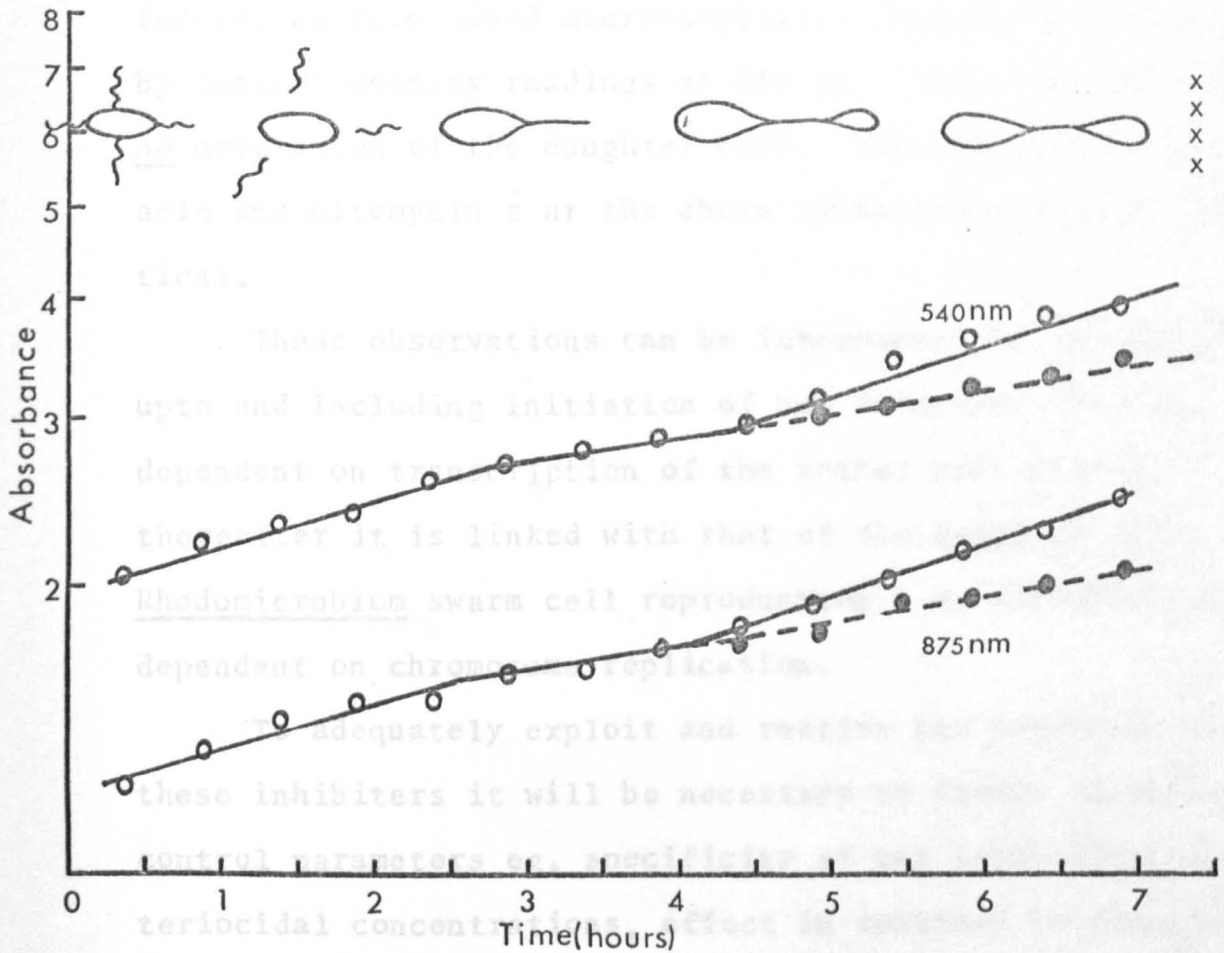


Figure 4.19

Effect of nalidixic acid (50 $\mu\text{g/ml}$) (●) and mitomycin c (5 $\mu\text{g/ml}$) (●) on synchronous growth of Rhodospirillum swarm cells (○).

tially growing vegetative cells in light (Figure 4.4). These 'wort-like growths' were observed in cells locked within a chained sequence, which may have physiological significance could not be explained without the study of ultrathin sections.

Patterns of DNA replication in Rhodospirillum rubrum have been studied extensively in the discoidal bacterium Rhodospirillum rubrum (Dagnen and Newton, 1977, 1 and 21-22).

The initial growth sequences i.e. maturation of the swarm cell, filament formation and bud formation were unaffected, as determined microscopically (morphologically) and by optical density readings at 540 nm. There was, however, no maturation of the daughter cell. The effects of nalidixic acid and mitomycin c at the above concentrations were identical.

These observations can be interpreted as showing that upto and including initiation of bud formation the cell is dependent on transcription of the mother cell genome, thereafter it is linked with that of the daughter cell. Rhodomicrobium swarm cell reproduction i.e. divisions, is dependent on chromosome replication.

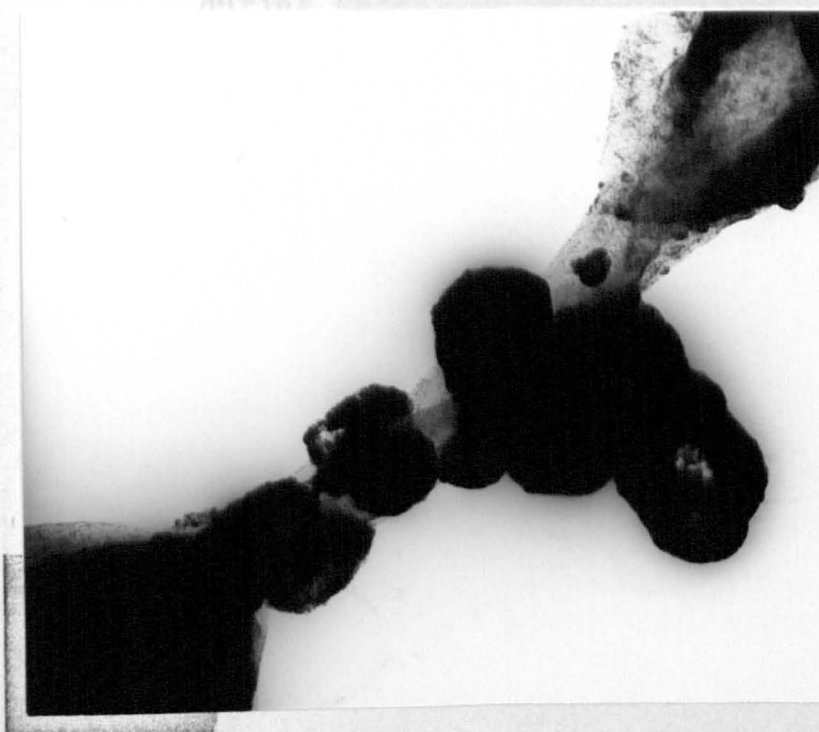
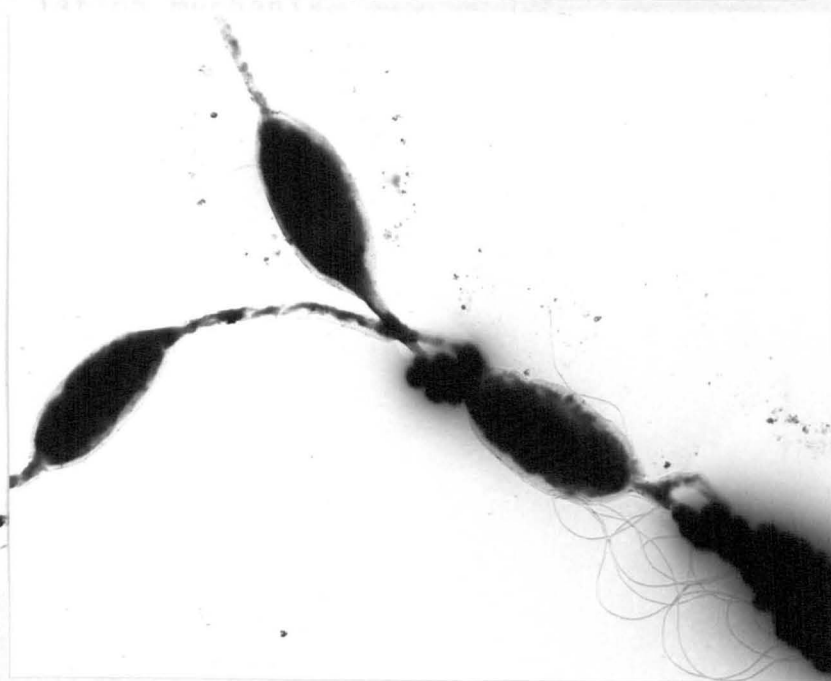
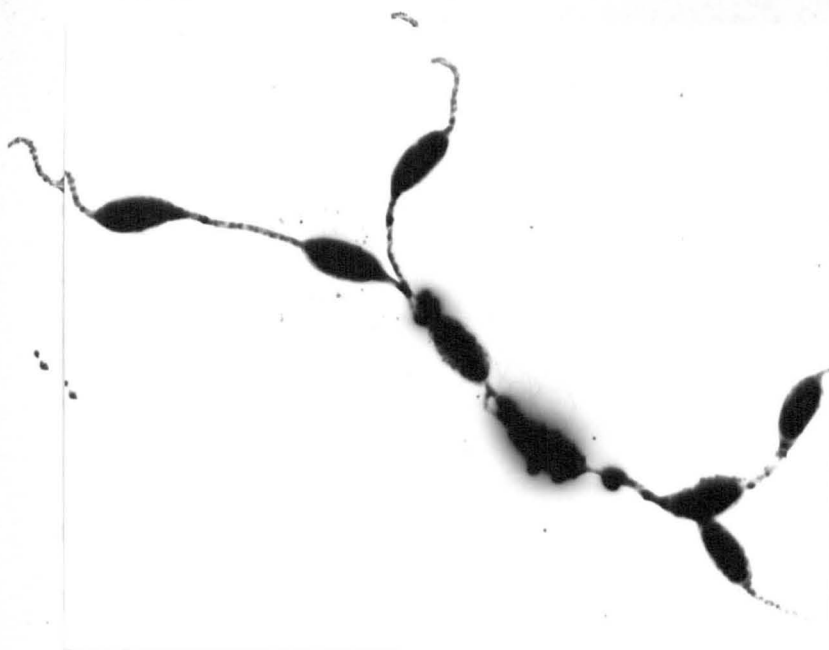
To adequately exploit and realise the potential of these inhibitors it will be necessary to firmly establish control parameters eg. specificity of the inhibition, bacteriocidal concentrations, effect in relation to time, etc. These were not examined in detail during the present work but it is obviously an approach to differential control mechanism worthy of detailed analysis and exploitation.

Nalidixic acid at a concentration of 150 µg/ml was found to cause morphological aberrations to exponentially growing vegetative cells in liquid culture (plate 4.4). These 'wort-like growths' were extensive on cells locked within a chained sequence, their derivation or physiological significance could not be determined without the study of ultrathin sections.

Patterns of DNA replication and development have been studied extensively in the dimorphic bacterium Caulobacter crescentus (Degnen and Newton, 1972, 1 and 2; Shapiro et al,

Plate 4.4

Gold-palladium shadowed electron micrographs of
Rhodomicrobium vegetative cells incubated in the
presence of 50 µg/ml of nalidixic acid.



1971). To explain the DNA 'cycles' of swarm and stalked cells Degnen and Newton (1972,1) have suggested that there is a structural requirement for the initiation of DNA replication and that in the stalked cell this is satisfied at division while in the swarm cell further development is required i.e. DNA synthesis is regulated in a special way to accommodate the differential pattern of growth. The above experiments on Rhodomicrobium indicate that a similar regulation mechanism may operate in swarm cells in that within the temporal sequence of differential events DNA synthesis occurs at a precise and well defined point in the cycle. Additionally the results suggest that cell division is coupled to the completion of chromosome replication (nalidixic acid, mitomycin c). The immediate questions raised therefore are, what mechanism controls the timing of initiation of chromosome replication, and how is initiation related to development? Experimentally these questions may be approached from two viewpoints:

- i) Is the temporal progression of events occurring during bacterial differentiation controlled by regulator gene products?
- or ii) Is the differential cycle like a biosynthetic pathway where one event must follow another?

The Rhodomicrobium life cycle, defined in synchronous swarm cell populations, includes a sequential series of both morphological and physiological changes that occur at specific times in the cell cycle. These distinct cellular characteristics peculiar to this organism have been defined in the above experiments and observations and may be summarised as follows:

1. All swarm cells are motile and immature.
2. Synchronous shedding of peritrichous flagella.
3. Initiation of synthesis of the complex lamellar membrane system i.e. induction of aminolaevulinate synthetase.
4. Initiation of DNA synthesis i.e. chromosome replication.
5. Maturation, ceasation of chlorophyll synthesis i.e. repression of swarm cell aminolaevulinate synthetase.
6. Filament formation.
7. Daughter bud formation.
8. Initiation of daughter lamellar membrane system i.e. induction of new, daughter specified aminolaevulinate synthetas.
9. Division i.e. filament 'plug' formation.

In essence Rhodomicrobium swarm cell populations are dependent on the irreversible differentiation of a flagellated swarm cell to a mature filamented cell. The system can be defined precisely by specific markers throughout the cycle and is therefore open to experimentation i.e. the questions relating to control mechanisms can be approached systematically by established techniques.

In comparison to the differential model based on Caulobacter (Shapiro et al, 1971; Degnen and Newton, 1972) and Hyphomicrobium (Moore and Hirsch, 1973) the developmental cycle of Rhodomicrobium swarm cells is in most respects comparable but has several distinct and unique advantages.

a) Maturation of the swarm cell i.e. period prior to filament synthesis. During the sequential events applicable to this stage in development the maturation of the Rhodomicrobium swarm cell can be followed accurately and precisely, not only by flagellar shedding and initiation of DNA synthesis, but by monitoring chlorophyll synthesis in two ways:

i) incorporation of labelled aminolaevulinate.

ii) assaying the induction and repression of a specific enzyme, aminolaevulinate synthetase.

This is characteristic of no other procaryotic differential system. Indeed experimental difficulties are arising with both Caulobacter and Hyphomicrobium differentiation systems specifically in relation to maturation of the swarm cell. The difference in the time required for the replication of the swarm and stalked cell in Caulobacter has repeatedly and erroneously been attributed to the time needed by the swarm cell to synthesise a stalk (Shapiro et al, 1971, Degnen and Newton, 1972¹). Staley and Jordan (1973) have indicated that after division the swarm cell and the stalked mother cell must synthesise stalk extensions. Therefore the maturation processes of the swarm cell are more complex than has been recognised by previous workers. Unlike the de novo chlorophyll synthesis in Rhodomicrobium there is as yet no readily available physiological marker associated with initiation or completion of the maturation process in either the Caulobacter swarm cell or the Hyphomicrobium swarm cell. Moore and Hirsch (1973) encountered serious problems in that they have found a long and significant lag period between swarm cell formation and initiation of filament synthesis. They have no means of assaying the lag physiologically and this may explain the poor synchronization obtained with this system.

b) Genetics will undoubtedly play a leading roll in unravelling the intricacies of the differentail model systems. To this end temperature sensitive mutants, in conjunction with established genetic techniques will be of considerable importance. However, the growth requirements and properties of

Rhodomicrobium permits the isolation of physiological mutants by virtue of the fact that growth can be sustained under photosynthetic (anaerobic) or non-photosynthetic (aerobic) conditions. No other differential system yet described offers this type of potential.

c) Recent studies with the coliphages T4, T7 and T3 suggests that DNA dependent RNA polymerase is involved in control of differential transcription (Travers, 1970; Chamberlin et al, 1970; Maitra, 1971) eg. during T4 development several changes have been reported to occur: a rapid loss of the σ subunit, (Walter et al, 1968; Bautz et al, 1969) an increase in the molecular weight of the α subunit (Seifert et al, 1969), attachment of 5^1 -AMP to the α subunit (Goff and Weber, 1970), and a change in the net charge of the β subunit (Travers, 1970). These differences alter the specific activities and stability but most importantly the template specificities of the enzyme. Another example of programmed transcription now actively being investigated is sporulation of Bacillus subtilis (Bautz, 1972); early in sporulation there is the loss of the vegetative σ factor from the RNA polymerase which causes a change in template specificity (Greenleaf and Losick, 1973). The above are however non-obligate differential systems.

The idea of multiple changes in RNA polymerase for selective transcriptional control in obligate differentiation has been investigated by Bendis and Shapiro (1973) using the Caulobacter system. However, the enzyme isolated from the swarm cell and the differentiated stalked cell appeared identical in subunit structure and template specificity.

In the context of transcriptional control being regulated by the DNA dependent RNA polymerase, Rhodomicrobium offers a unique system for the investigation of such a mechanism. Not only is there the obligate defined differentiation of swarm cell to stalked mother cell there is also, within the same organism, non-obligate exospore formation and germination. Therefore by synchronisation of either cell type (swarm cell or exospore) one can study the role of transcriptional control by the RNA polymerase in both obligate and non-obligate, sequential differential events.

d) Mortality.

'Normal' bacterial growth and division involves a distribution of 'old' and 'new' cellular material between the two derivative daughter cells. There can therefore never be any question of the cell aging by more than the length of the growth cycle. Cellular systems such as Caulobacter, Hyphomicrobium and in particular Rhodomicrobium however, introduce into the procaryotic realm the phenomenon of mortality i.e. the daughter cells involve de novo synthesis of cell material, in consequence the mother cell ages with each generation. This inherent limitation on the mother cell, and the germinating exospore, may explain the limitation on the number of daughter cells which can be derived from any one mother. The potential to exploit the Rhodomicrobium system at the molecular level for protein, DNA, RNA turnover within the aging differentiated cell is immense and unique to this organism. There is also the additional question as to aging of the hereditary material and the consequences of this.

Figure 4.20 summerises the known physiological and

Figure 4.20

Schematic representation of the developmental sequences of Rhodospirillum rubrum (Rm5).

lm	loss of motility (flagellar shedding)
ala s <u>i</u>	aminolaevulinate synthetase induction
i DNA	initiation of DNA synthesis
ala s <u>r</u>	aminolaevulinate synthetase repression
fs	filament formation
bf	bud formation
cf	constriction or 'plug' formation.

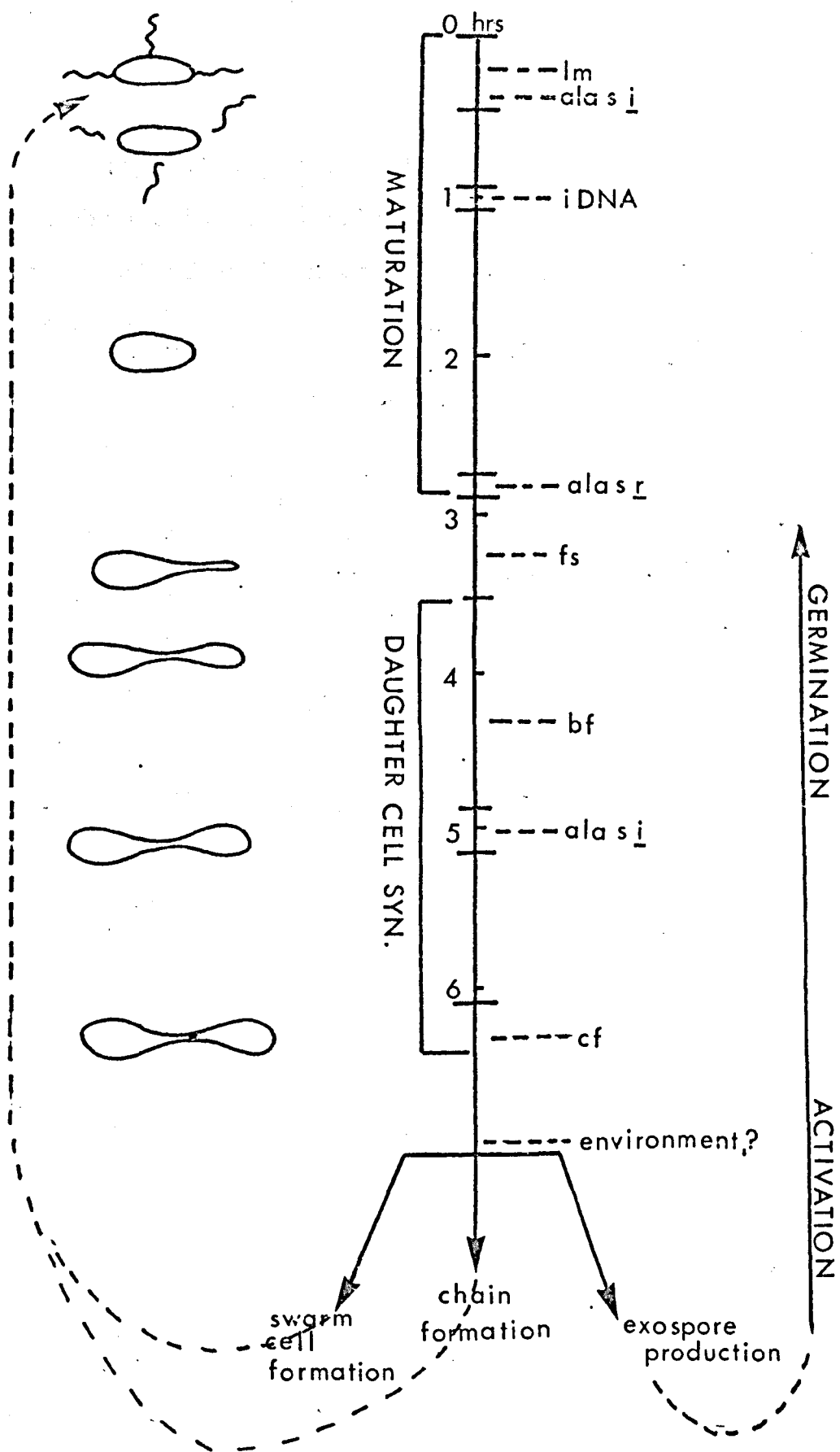


Figure 4.20

morphological aspects of the Rhodomicrobium growth cycle and from this it is relatively simple to envisage the potential of such an organism in the realm of differential research.

Section 5.

Qualitative Investigation of the DNA of the

"Budding" Bacteria.

Section 5.1Introduction

DNA from a single bacterial species commonly shows a unimodal distribution of nucleotides and a relatively homogeneous composition throughout its length (Sueoko, Harmur and Doty, 1959; Rolfe and Meselson, 1959). However, many bacteria possess other genetic elements within the cell e.g. plasmids (Maynell, 1971). The existence of a plasmid is usually recognised by the function it determines e.g. drug resistance (Watanabe, 1963); colicin production (Nomura, 1967); ϕ x factor activity (Hayes, 1953) or virus production. The similarities possessed by bacterial plasmids, particularly their autonomous existence and self-replication within the cell, have been recognised as being more significant than their differences e.g. origin, molecular weight, base composition. Moreover, many of their properties are shared with the extranuclear DNA associated with subcellular organelles of eukaryotic cells, e.g. chloroplasts, mitochondria and kinetoplasts.

With few exceptions the investigation of bacterial plasmids has centered on the Enterobacteriaceae, Escherichia coli, Salmonella, Shigella and Klebsiella species, all primarily from a genetical standpoint. Suyama and Gibson, (1966) however, seeking a possible parallel with the autonomous DNA of the eukaryotic chloroplasts investigated the possibility of extrachromosomal or satellite DNA being present in photosynthetic procaryotes where the photosynthetic apparatus is located in the membrane invaginations e.g. thylakoids (Schachman, Pardee, and Stanier, 1952). Although these workers detected satellite DNA in some species

of photosynthetic bacteria, their origin and function remained unknown.

Shapiro et al (1970) reported the presence of extrachromosomal DNA in the dimorphic microorganism Caulobacter. The unique feature of this satellite DNA was that it could only be detected in the stalked mother cells and not in the motile swarm cells. The inference is that the specificity of the satellite DNA may accord it a differentiation function during the life cycle of the organism.

The DNA of the majority of phototrophic bacteria (Mandel et al, 1971) and of the budding filamentous Hyphomicrobium species (Mandel, Hirsch and Conti, 1972) have been examined by CsCl density gradient centrifugation. In neither investigation was any species of satellite DNA encountered. Satellite bands occasionally found were shown to be from contaminant organisms. The satellite DNA having a buoyant density corresponding to that of the isolated contaminant.

Until recently the physical analysis of plasmids has been dominated by two unrelated techniques which have given results of striking agreement. The first involves various forms of ultracentrifugal analysis, notably, density gradient centrifugation in CsCl by which macromolecules are separated according to their density and zone sedimentation in preformed gradients where macromolecules are separated according to their shape and molecular weight. The second technique is electron microscopy which has enabled the indirect estimates of size and configuration accessed from centrifugation to be verified by observation.

Density gradient centrifugation in CsCl is a very sensitive technique when carried out in an analytical ultracentrifuge but suffers from the disadvantage that it fails to reveal the differences between plasmid and chromosomal DNAs which have almost the same G+C content and hence the same buoyant density resulting in the large chromosomal band being superimposed on the plasmid fraction which is entirely masked. A more sensitive technique and one which poses fewer problems of interpretation has been used by Aaij and Borst (1972) who have compared the electrophoretic mobility of linear duplex and circular duplex DNAs in agarose gels using the intercalating dye ethidium bromide to allow immediate visualization of the fractionated DNA bands. Their results show that electrophoresis provides a sensitive method for the separation of closed and open circular DNAs and that the system will in fact separate open circular and linear DNAs of the same molecular weights up to 10×10^6 daltons. Flint and Harrington (1972) extended this line of research in investigating DNAs of molecular weights ranging from 8.5×10^4 to 2×10^8 daltons. Harley et al (1973) showed that electrophoretic analysis of DNA allowed differentiation of the three structural classes, single stranded, double stranded linear and double stranded circular. Both used polyacrylamide or polyacrylamide/starch gels in preference to agarose gels but nevertheless demonstrated the advantages and versatility of electrophoresis as a method of analysis and characterisation of DNA species.

The common morphological and physiological characteristics of the known budding bacteria, in particular the possession of obligate differential life cycles, prompted a qualitative

study of the DNAs of this group of microorganisms. To check the findings of Shapiro et al (1970) and to ascertain the possibility of these organisms possessing autonomous satellite DNA closely associated with the principle differentiation step, the techniques of analytical density gradient ultracentrifugation and of gel electrophoresis were employed.

Section 5.2Materials and Methods.

a) Source of organisms and culture medium.

Table 5.1

<u>Organisms.</u>	<u>Source</u>	<u>Culture medium.</u>
<u>Rhodomicrobium</u> <u>vannielii</u> (type strain)	R. Whittenbury Univ.of Warwick.	PYR medium ¹
<u>Rhodopseudomonas</u> <u>palustris</u>	Enrichment ¹	SPR medium ¹
<u>Rhodomicrobium</u> (Rm5)	Enrichment ¹	PYR medium ¹
<u>Rhodopseudomonas</u> <u>viridis</u>	Enrichment ¹ N. Pfennig, Univ.of Gottingen.	SPR medium ¹ supplemented with 0.1g/litre yeast extract.
<u>Rhodopseudomonas</u> <u>acidophila</u>	Enrichment ¹	SPR medium (pH 5.5)
<u>Caulobacter</u>	Enrichment ²	G + Y medium ²
<u>Nitrobacter</u>	A. Smith, Univ.of Aberystwyth	Smith and Woare (1968)
'Mushroom' budding bacteria	R. Whittenbury B.V.M.Gorenko,Moscow	Whittenbury and Nicol1 (1971)
<u>Hyphomicrobium</u>	Enrichment ² W. Harder, Univ. of Groningen	Attwood and Harder (1972)
<u>Escherichia coli</u> strains	S.B. Primrose, Univ.of Warwick	Nutrient Broth
Phage T7 EC1	S.B. Primrose, Univ. of Warwick.	

1. section 3.2

2. section 2.2

b) Properties of Escherichia coli strains.

Table 5.2

<u>Escherichia coli</u>	<u>No. of plasmids</u>	<u>Mol. Weight (daltons)</u>
M9	3	9.5 x 10 ⁶ 65 x 10 ⁶ 118 x 10 ⁶
C603	-	-
M7	1	65 x 10 ⁶
R7	(<u>Klebsiella</u> DNA integrated)	65 x 10 ⁶ 118 x 10 ⁶

reference. Dixon, Postgate and Primrose (1974).

c) Solutions.Standard Saline Citrate (SSC)

Ten times concentrated standard saline citrate (10 x SSC) consists of 1.5M NaCl + 0.15M Na citrate adjusted to pH 7.0. Other concentrations of SSC were prepared by dilution without pH adjustment.

Tris HCl buffer.

Tris HCl buffer is a 0.2M solution of Trizma base (Tris (hydroxymethyl) amino methane) adjusted to pH 7.2 with HCl.

TES buffer.

TES buffer is 0.05M Tris pH 7.2 + 0.001M EDTA + 0.1M NaCl.

Phage buffer.

Previously described (section 3.2).

d) Phage preparation and purification.Phage T7 and EC1.

2 litres of Escherichia coli strain H was grown at 30°C till a density of 1×10^8 cells/ml was reached. The cultures were seeded with T7 or EC1 at a multiplicity of infection of 0.1 and incubated until lysis was observed.

Phage were precipitated by the addition of sodium chloride (29.5 gm/litre) and polyethylene glycol (80.0gm/litre) to the culture lysate with overnight storage at 4°C. The lysate was centrifuged (4,000g) and the pellet resuspended in a minimum volume of 'phage buffer. The resulting phage suspension was layered onto a discontinuous CsCl gradient composed of 1 ml of each of CsCl solutions of 1.3; 1.4; 1.5; and 1.6; g/cc³.and centrifuged in an MSE 3 x 6.5 swing out rotor at 40,000 rpm for 2 hrs. The resulting phage band was collected by puncturing the polycarbonate centrifuge tube at the bottom and collecting the appropriate fraction. CsCl was removed by overnight dialysis against phage buffer at 4°C with at least two changes of buffer. Purified phage was stored in phage buffer at 4°C.

Phage λ .

Escherichia coli strain SA270 which is lysogenic for the thermoinducible phage λ CK 857 was grown at 30°C until a density of 3×10^8 cells/ml was reached. The culture was then incubated at 42°C for 30 minutes to inactivate the λ repressor and reincubated at 30°C until clearing occurred. Phage precipitation and purification was identical to that for EC1 and T7.

e) Phage DNA extraction.

DNA was extracted from the bacteriophage by the method of Rovre and Szybalski (1971).

f) Bacterial DNA extraction and purification.

Bacterial cultures from the late exponential growth phase were harvested by centrifugation at 20,000g for 30 minutes and washed in TES buffer (pH 7.1). Freshly prepared

lysozyme was added to a final concentration of 4mg/ml and the culture incubated at 37°C for 1-6 hrs. The addition of sodium lauryl sarcosinate to a final concentration of 2% (w/v) brought about lysis.

Separation of DNA from cellular debris, RNA and protein was achieved by centrifuging the lysate at 120,000g for 36 hrs. (8 x 25ml MSE titanium rotor) in a linear CsCl gradient of mean density 1.71 gm/cc³. 25 drop fractions were collected by puncturing the bottom of the centrifuge tube and dialysed overnight at 4°C against SSC. Sample purity was determined by reading A_{280}/A_{260} and the DNA concentration calculated from the extinction coefficient.

Purification was carried out by the addition of 1 ml redistilled, water saturated phenol per 2 ml sample and rotating slowly for 10 minutes. After centrifugation to separate the phases the upper aqueous phase was dialysed against 0.1 SSC. DNA samples were stored in 0.1 SSC over chloroform at 4°C.

As an alternative method of preparing a cell lysate the washed cell suspension was subjected to 9,000 psi at 4°C in an Aminco French pressure cell. The major disadvantage of this preparative procedure was the excessive shearing forces to which the DNA was subjected. For this reason the enzymatic procedure was used preferentially.

As a control to ensure that there was no loss of extrachromosomal DNA during the preparative centrifugation step the preparative procedure of Mandel (1966) was used to double check the methodology.

g) Equilibrium sedimentation in a CsCl density gradient.

Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge for at least 22 hrs. at 44,000 rev/min. and 25°C in a cell with a 12mm 4° Kel F centrepiece. Photographs were taken using ultra-violet absorption optics and the films examined either by photographic enlargement or with a Joyce-Loeble recording microdensitometer (figure 5.1). DNA from Escherichia coli strain M7, $\rho=1.7092 \text{ gm/cc}^3$, phage $\rho=1.7093 \text{ gm/cc}^3$ and phage T7 $\rho=1.710 \text{ gm/cc}^3$ were used as markers and buoyant densities were calculated by the method of Mandel et al(1968).

The buoyant density of each sample in neutral CsCl was determined at least twice.

For analytical runs in alkaline CsCl, samples were prepared in fresh 0.1M K_2HPO_4 which had been adjusted to pH 12.4 with 50% (w/v) KOH.

h) Digestion of DNA.

Deoxyribonuclease digestion of DNA (20-50 $\mu\text{g/ml}$) was at 37°C for 1 hr. in the presence of 10 units/ml of Micrococcal deoxyribonuclease (grade IV, Sigma).

For heat denaturation, DNA in 0.1 SSC contained in a thin walled glass tube was placed in a boiling water bath for 5 minutes and then quickly cooled.

i) Gel electrophoresis of DNA.

The gel electrophoresis of DNA was carried out as described by Aaij and Borst (1972).

Agarose gels were prepared by dissolving 1% (w/v) agarose (Sigma electrophoresis grade) in a hot solution of 0.04M Tris-HCl-0.02M sodium acetate-2mM disodium EDTA, pH 7.7 (electrophoresis buffer F). After cooling the

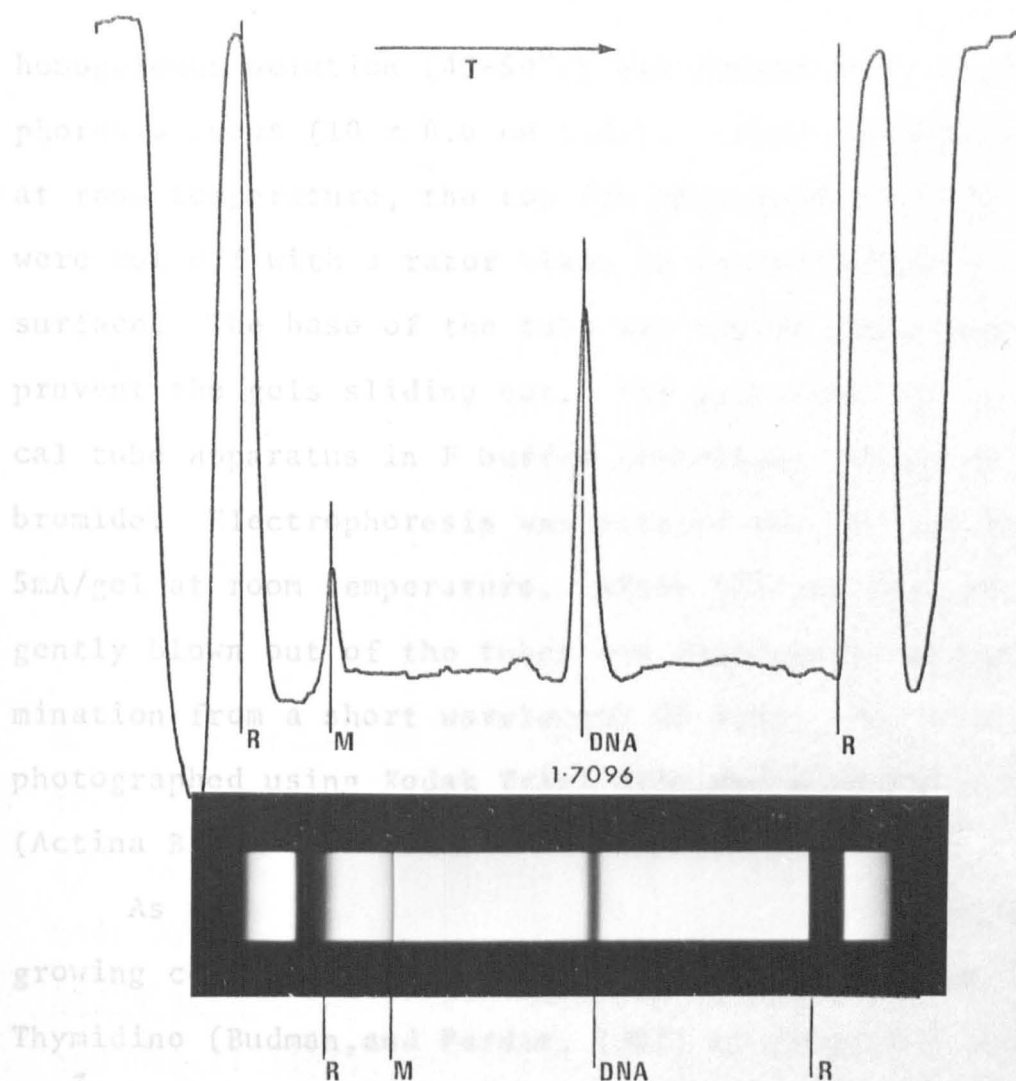


Figure 5.1

Microdensitometer tracing of ultraviolet absorption photograph (insert) of 1.0 μg T7 DNA in neutral CsCl . Centrifugation at 44,000rpm for 21 hrs. at 25°C .

R Reference peaks.

M Meniscus

DNA Deoxyribonucleic Acid

T Trace direction

Buoyant density (ρ) of T7 DNA, 1.7096.

homogeneous solution (45-50°C) was poured into electrophoresis tubes (10 x 0.6 cm i.d.). After gelatinization at room temperature, the top few millimeters of the gels were cut off with a razor blade to ensure a flat gel surface. The base of the tube was sealed using muslin to prevent the gels sliding out. The gels were run in vertical tube apparatus in F buffer containing 100 µg/ml ethidium bromide. Electrophoresis was carried out for 1-2 hrs. at 5mA/gel at room temperature. After the run the gels were gently blown out of the tubes and examined by direct illumination from a short wavelength UV lamp. The gels were photographed using Kodak Tri-X film and a yellow filter (Actina BDB 49 X75mm).

As a control, tritium labelled DNA was prepared by growing cells in the presence of 10 µCi/ml (methyl-³H) Thymidine (Budman, and Pardee, 1967) or 10 µCi/ml Deoxy (8-³H) guanosine (Degnen and Newton, 1972). Extraction and purification procedures were as detailed above. Duplicate gels were either stained with ethidium bromide (100 µg/ml) or sliced into 1mm sections which were digested with 0.1ml hydrogen peroxide at 30°C for 2 hrs. and counted in triton toluene scintillant which contained 36 gm 2,5-diphenyl-oxazole and 0.45 gm 0,4-bis-(5 phenyloxazole-2-yl) benzene dissolved in 6 litres toluene plus 3 litres triton x-100.

Section 5.3Results and Discussion.

Mandel et al (1971) have published CsCl buoyant density measurements of the DNA G+C% (guanine plus cytosine) content of a large number of phototrophic bacteria. The following year Mandel, Hirsch and Conti (1972) presented the guanine plus cytosine mole percentages of hyphomicrobia "in order to provide a valid base for comparison with values obtained for the phototrophic bacteria", therefore floating the idea that the morphological and environmental similarities that characterise these different types of bacteria may be reflected in the similarities and homogeneity of their G+C%. The studies reported here present further data for such comparisons, based on budding as a 'distinctive' mode of growth and reproduction, and includes data on Nitrobacter; the recent 'mushroom' isolates of Whittenbury and Nicoll (1971) and Gorlenko (1972) (figure 5.2) and the spore forming species of Rhodomicrobium (section 3.3).

Satellite DNA of Caulobacter species?

These studies were not instigated solely to make comparative G+C% determinations but also to detect any discrete extrachromosomal plasmid formation; these having been reported in stalked cells but not swarm cells of Caulobacter crescentus. The implication was that they played some role in the differentiation process (Shapiro and Agabian-Kashishian, 1970) (figure 5.3).

Two techniques were employed in an attempt to amplify and verify the findings of Shapiro and Agabian-Kashishian (1970).

- a) equilibrium sedimentation in CsCl density gradients.
- b) gel electrophoresis (Aaij and Borst, 1972).

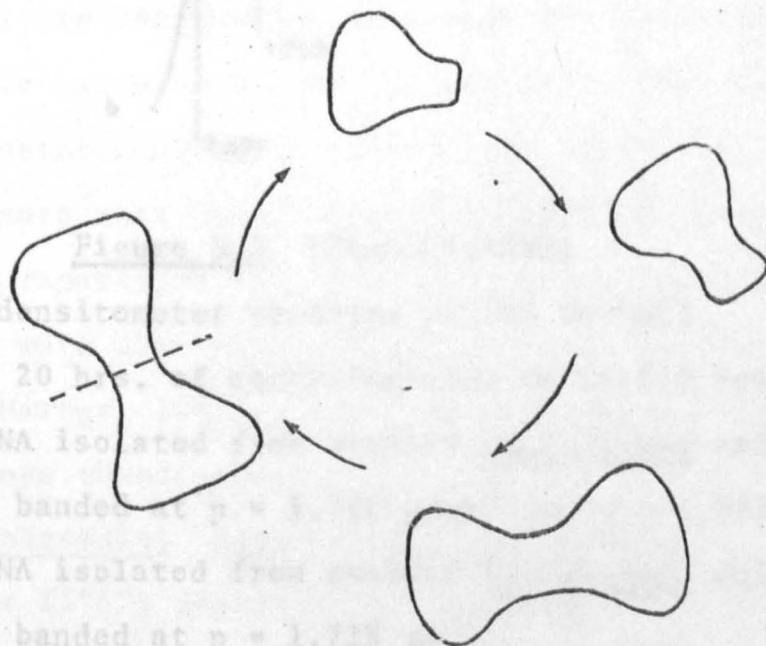
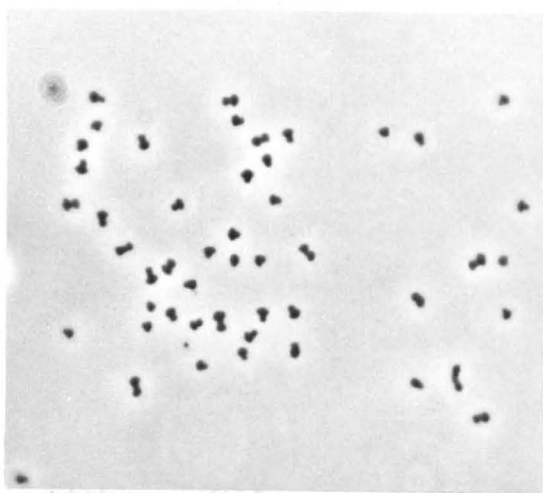


Figure 5.2

Phase contrast photomicrograph (x 1100) and diagrammatic representation of the life cycle of a 'mushroom-shaped' budding bacterium isolated by Whittenbury and Nicoll (1971).

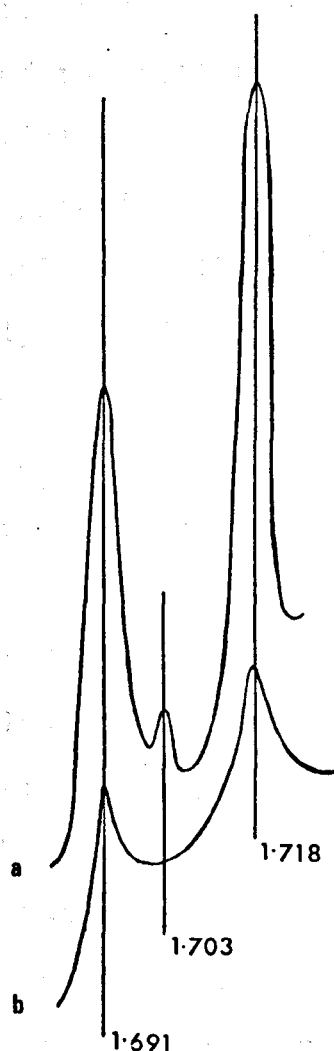


Figure 5.3 (Shapiro, 1970)

Microdensitometer readings of DNA in CsCl
after 20 hrs. of centrifugation at 44,770 rev/min.

a) DNA isolated from stalked Caulobacter cells
which banded at $p = 1.718 \text{ g/cm}^3$ and $p = 1.703 \text{ g/cm}^3$.

b) DNA isolated from swarmer Caulobacter cells
which banded at $p = 1.718 \text{ g/cm}^3$.

Clostridium perfringens ($p = 1.691 \text{ g/cm}^3$) was used
as a reference marker.

CsCl density gradient centrifugation of *Caulobacter* DNA.

DNA was extracted from *Caulobacter* cultures enriched in one or other of the cell types:

- i) late exponential growth phase, heterogeneous cultures which contained approximately 20% of the cell population as swarm cells, 80% as stalked mother cells.
- ii) approximately 80% of the cell population as swimmers; enriched by the column separation technique (section 4.2).

In neither case was a satellite band detected, nor was there any detectable difference in the buoyant densities (figure 5.4).

Analysis of DNA samples by this technique gives rise to two problems leading to possible misinterpretation of results. These are the masking of the satellite band by the primary DNA band, and, secondly, insufficient concentration of the satellite DNA species to permit its detection. Since no steps were taken to enrich for satellite DNA, alteration of one parameter conversely effects the other.

To ensure that loss of satellite DNA was not occurring during the preparative CsCl density gradient centrifugation, DNA samples were also prepared by the phenol extraction technique (Marmur, 1961). There were no detectable changes from the above observations.

Gel electrophoresis; Agarose:Ethidium bromide.

Thorne (1967) described the separation of closed and open circular forms of polyoma virus DNA by gel electrophoresis through agar gels. Aaij and Borst (1972) improved the method by using 0.6-1.0% (w/v) agarose gels and low concentrations of ethidium in the electrophoresis buffer. Their results show that electrophoresis provides a simple

Microdensitometer tracings and UV absorption photographs of Caulobacter DNA (enriched in stalked cells) after centrifugation at 44,000 rev/min. at 25°C for 20 hrs.

A. 1.5 µg DNA.

B. 1.0 µg DNA.

R. reference peaks

M. meniscus

(A) DNA $p = 1.7204$

(B) DNA $p = 1.7209$.

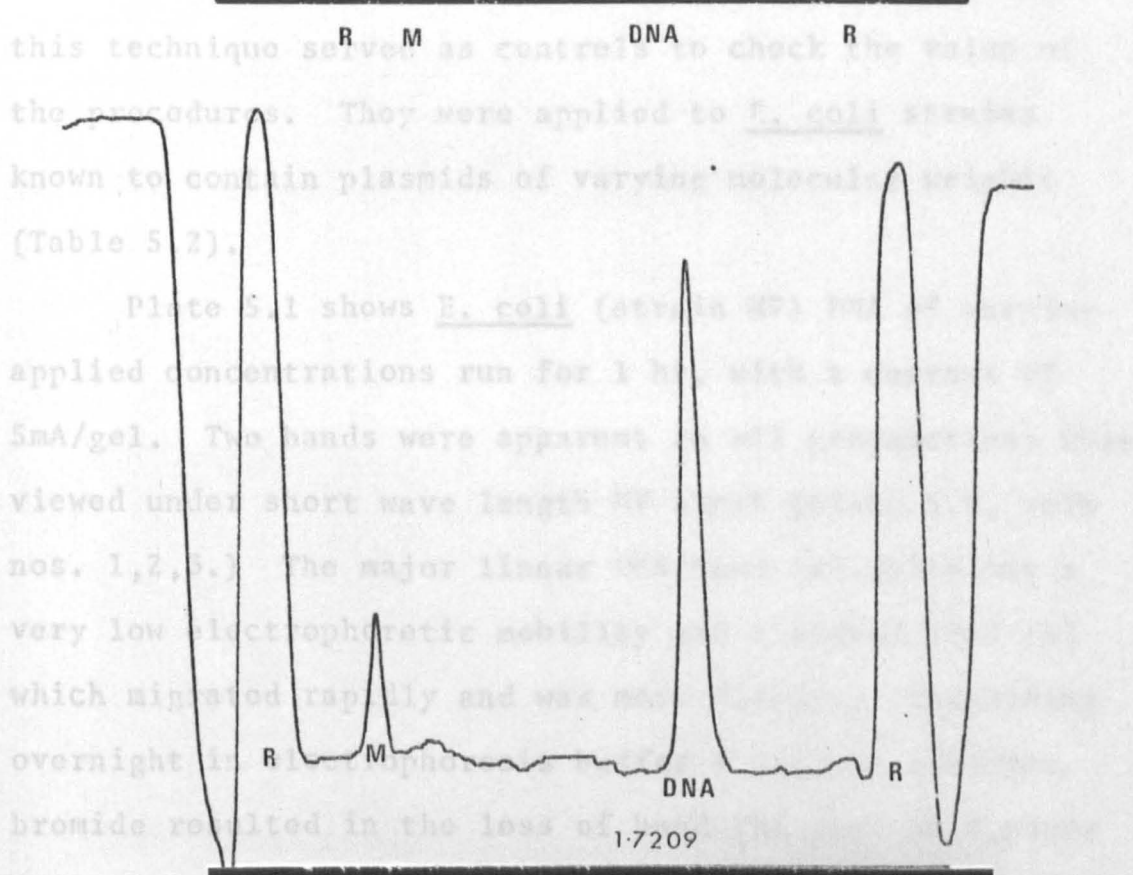
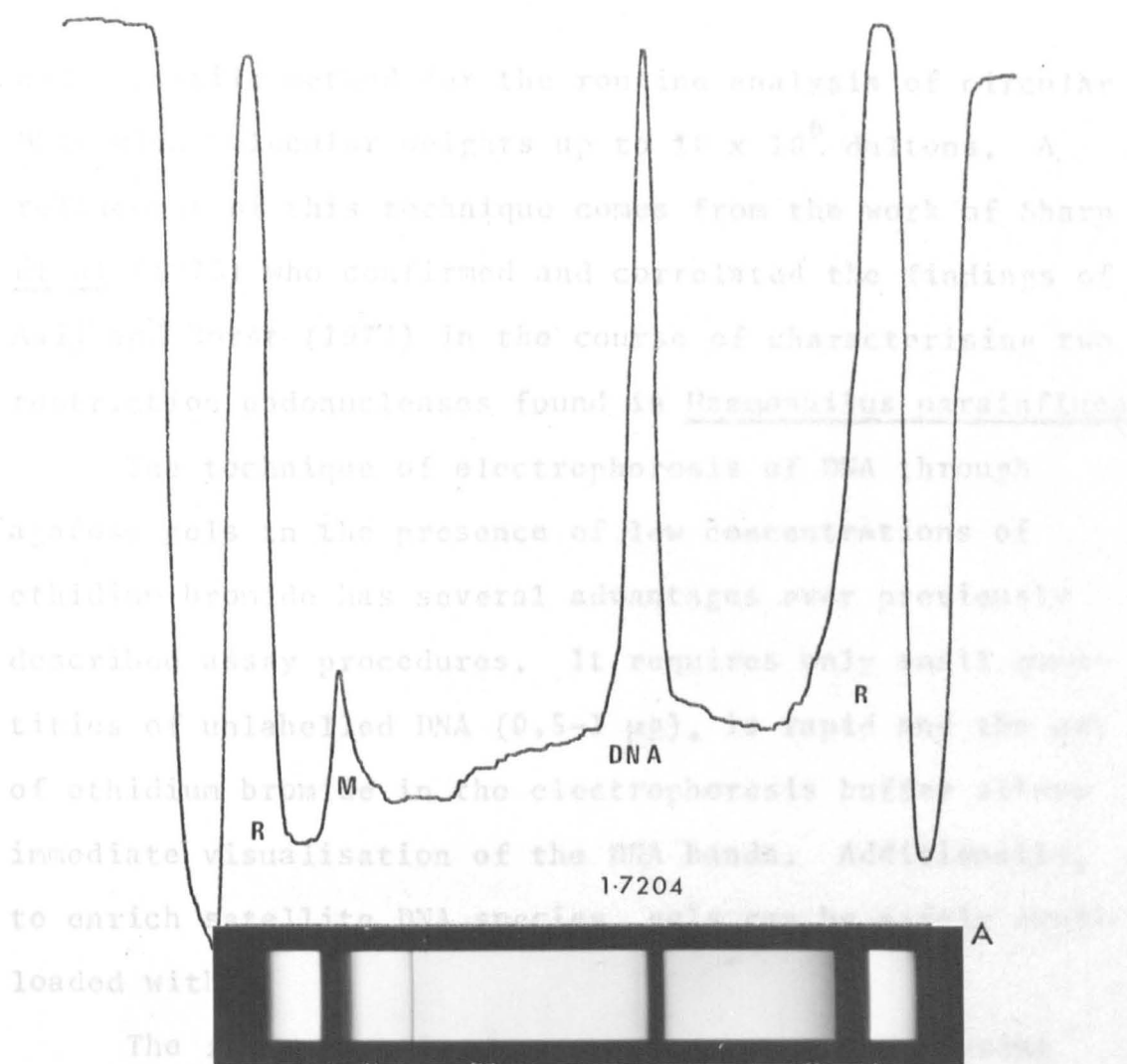


Figure 5.4

and versatile method for the routine analysis of circular DNAs with molecular weights up to 10×10^6 daltons. A refinement of this technique comes from the work of Sharp et al (1973) who confirmed and correlated the findings of Aaij and Borst (1972) in the course of characterising two restriction endonucleases found in Haemophilus parainfluenzae.

The technique of electrophoresis of DNA through agarose gels in the presence of low concentrations of ethidium bromide has several advantages over previously described assay procedures. It requires only small quantities of unlabelled DNA (0.5-1 μ g), is rapid and the use of ethidium bromide in the electrophoresis buffer allows immediate visualisation of the DNA bands. Additionally, to enrich satellite DNA species, gels can be safely overloaded with linear DNA.

The first series of experiments undertaken using this technique served as controls to check the value of the procedures. They were applied to E. coli strains known to contain plasmids of varying molecular weights (Table 5.2).

Plate 5.1 shows E. coli (strain M7) DNA of varying applied concentrations run for 1 hr. with a current of 5mA/gel. Two bands were apparent in all preparations when viewed under short wave length UV light (plate 5.1, gels nos. 1,2,3.) The major linear DNA band (a) which has a very low electrophoretic mobility and a second band (b) which migrated rapidly and was more diffuse. Destaining overnight in electrophoresis buffer F lacking ethidium bromide resulted in the loss of band (b) (gel no.4, plate 5.1).

Plate 5.1 Agarose gel electrophoresis of varying applied concentrations of Escherichia coli (M7) DNA.

Current of 5 mA/gel for 1 hr.

1. 0.5 µg M7 DNA.
2. 1.5 µg M7 DNA.
3. 4 µg M7 DNA.
4. 1.5 µg M7 DNA destained overnight in electrophoresis buffer F lacking ethidium bromide.
 - a) Primary linear double stranded DNA.
 - b) Satellite species.

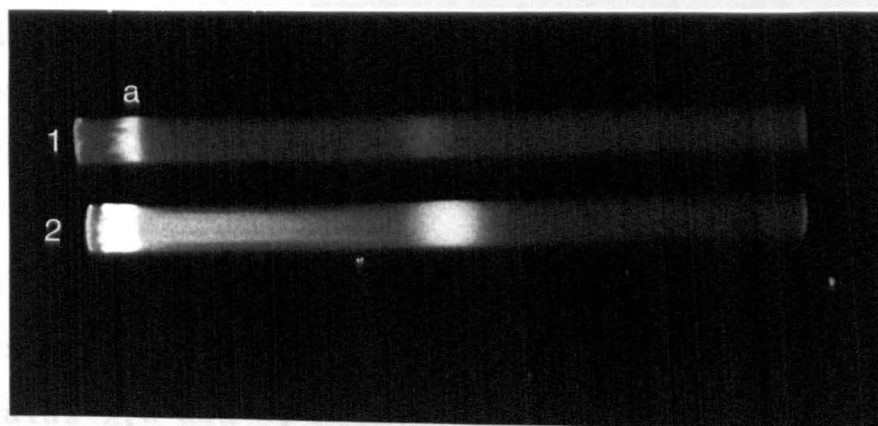
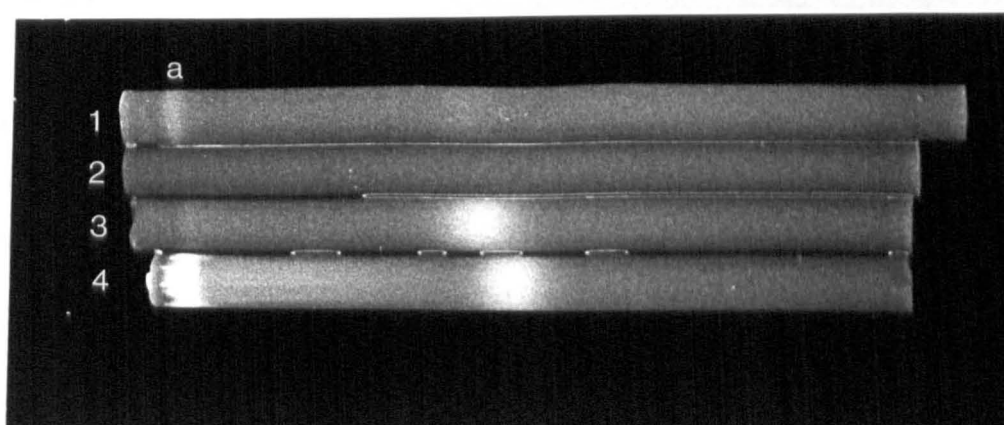
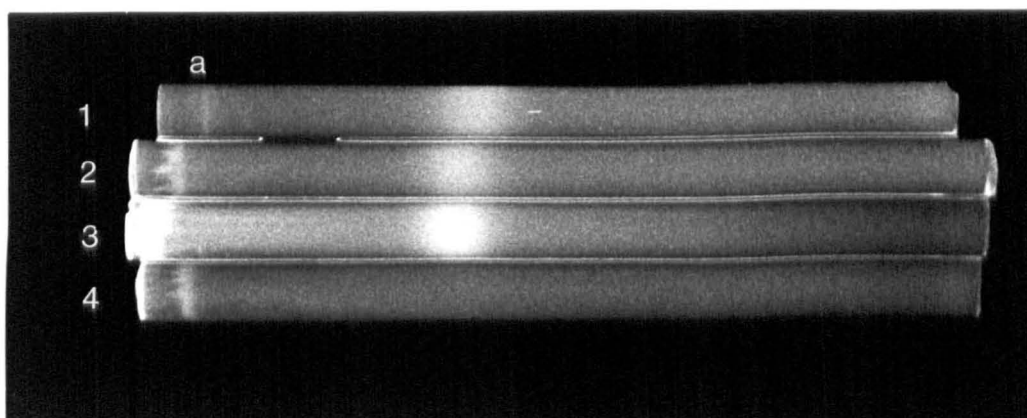
Plate 5.2 Agarose gel electrophoresis to determine the probably nature of band b.

Current of 5 mA/gel for 1 hr.

1. 1.5 µg E. coli(M7) DNA destained overnight.
2. Control gel lacking DNA but otherwise subjected to the normal procedure.
3. Sheared E. coli (M7) DNA.
4. 4 ug E. coli (M7) DNA.

Plate 5.3 Caulobacter and E. coli (M7) DNA analysed by agarose gel electrophoresis. Current of 5 mA/gel for 1 hr.

1. 3 µg Caulobacter DNA.
2. 4 µg E. coli (M7) DNA.



and Rhodomicrobium species. The
type examined i.e. strain 10-10-10

Plate 5.2 is representative of a sequence of experiments to determine the probable nature of band (b).

As mentioned above, overnight destaining results in the loss of the satellite (b) band (gel no.1, plate 5.2). If a control gel is run i.e. lacking a DNA sample, but otherwise subjected to the normal procedures, no bands are detected (gel no.2, plate 5.2). However if heavily sheared DNA is electrophoresed a very definite satellite (b) band is detected but only a very faint primary band (a) (gel no.3, plate 5.2) as opposed to the definite double band pattern present in untreated samples (gel no.4, plate 5.2).

These results in conjunction with the electrophoretic analysis of tritium labelled DNA samples (figures 5.6,5.8) tend to suggest band (b) is composed of oligonucleotides, hence the speed of movement and the rapidity of diffusion from the gel.

No plasmid DNAs could be detected in the E. coli strains examined by this technique. This was probably because they were of too high a molecular weight and hence inseparable from the main, linear DNA band. Alternatively there was too little present. However, to check the Caulobacter DNA samples these were run on the above electrophoretic system. All gave negative results (plate 5.3) irrespective of variation in current/gel or time of passage.

From these experiments it can be concluded that there are no extrachromosomal, circular DNAs of molecular weight less than 8×10^6 daltons present in Caulobacter, Hyphomicrobium strains X,C and G, Rhodopseudomonas acidophila, R. palustris and Rhodomicrobium strain Rm5, irrespective of the cell type examined i.e. mother or daughter cell.

Gel electrophoresis; Agarose:tritiated DNA samples.

E. coli strains M7, M9, C603, and R7 were grown in the presence of (methyl-³H) Thymidine, and Caulobacter in the presence of Deoxy (8-³H) guanosine. As a further check of the electrophoretic system these tritium labelled DNAs were applied to 1% (w/v) agarose gels, subjected to a current of 5 mA/gel for a period of 2 hrs, sliced into 1 mm sections, and assayed for radioactivity.

E. coli strains M7, C603 and R7 gave only single peaks of radioactivity (figure 5.5, 5.6) which correspond to the primary (a) bands found in similar gels where the DNA is complexed with ethidium bromide. Interference from oligonucleotide satellite band (b) is avoided by increasing the time of electrophoresis from 1-2 hrs., effectively eluting this component from the gel (counts from the oligonucleotides can, however, be observed at the base of several of the gels, as in figure 5.6). The sensitivity of this technique allowed the smallest of the M9 plasmids (9.5×10^6 daltons) to be detected (figure 5.6). It must be assumed that the other large molecular weight plasmids move with the major linear double stranded DNA band.

An increase in the time of electrophoresis from 2-4 hrs. resulted in the stretching of the linear DNA band and a loss, both of resolving power and a failure to detect the M9 plasmid, presumably because of dilution initially and finally by elution (figure 5.7).

Tritiated Caulobacter DNA, as in the ethidium:agarose system gave only a single peak, corresponding to the linear

high molecular weight DNA (figure 5.8) i.e. no plasmid was detected despite the ability to overload such gel systems.

Alkaline CsCl gradients.

Vinograd et al (1963) and Primrose (1971) have shown that single stranded DNA rich in thymine and guanine is denser than DNA deficient in thymine and guanine in alkaline CsCl gradients. Hence, centrifugation of DNA in CsCl gradients offers the possibility of detecting heterogeneity in base composition between the DNA strands. Vinograd et al (1966) and Crawford (1968) have also shown not only that at pH 12.5 linear double stranded DNA is separated into its constituent strands, but that double stranded, circular plasmid DNA cannot separate completely at high pH and therefore collapses upon itself to form a compact dense structure.

By subjecting the DNAs of Escherichia coli (R7) and Caulobacter to alkaline CsCl density centrifugation it was hoped, by the subsequent denaturation of the linear DNA, to enhance the intact plasmid DNA, essentially surmounting the problem of masking by DNAs of similar G+C contents.

Plate 5.4a of E. coli strain R7 DNA centrifuged under alkaline conditions shows two discrete bands, masked to a certain extent by denatured linear DNA but still discernable as separate entities. In contrast Caulobacter DNA exposed to similar conditions (Plate 5.4b) revealed only a single species.

The observations made on E. coli strain R7 DNA may be explained in two ways. Firstly, each band corresponds to single stranded linear DNA differing in guanine and/or

Figure 5.5

Agarose gel electrophoresis of 5 μ g tritiated Escherichia coli DNA from strains M7 and C603 showing single peaks of radioactivity

Figure 5.6

Agarose gel electrophoresis of 5 μ g tritiated Escherichia coli DNA from strains R7 and M9.

The smallest plasmid of M9 (9.5×10^6 daltons) is resolved (St) as are the interfering oligonucleotides (OL) at the base of the gel.

Figure 5.7

Agarose gel electrophoresis of 5 μ g DNA samples from E. coli strains C603 and M9.

A. 5 mA/gel for 2 hrs.

B. 5 mA/gel for 4 hrs.

Figure 5. 8

Caulobacter and E. coli (M7) DNA examined on 1% (w/v) agarose gels with a current of 5 mA/gel for 2 hrs.

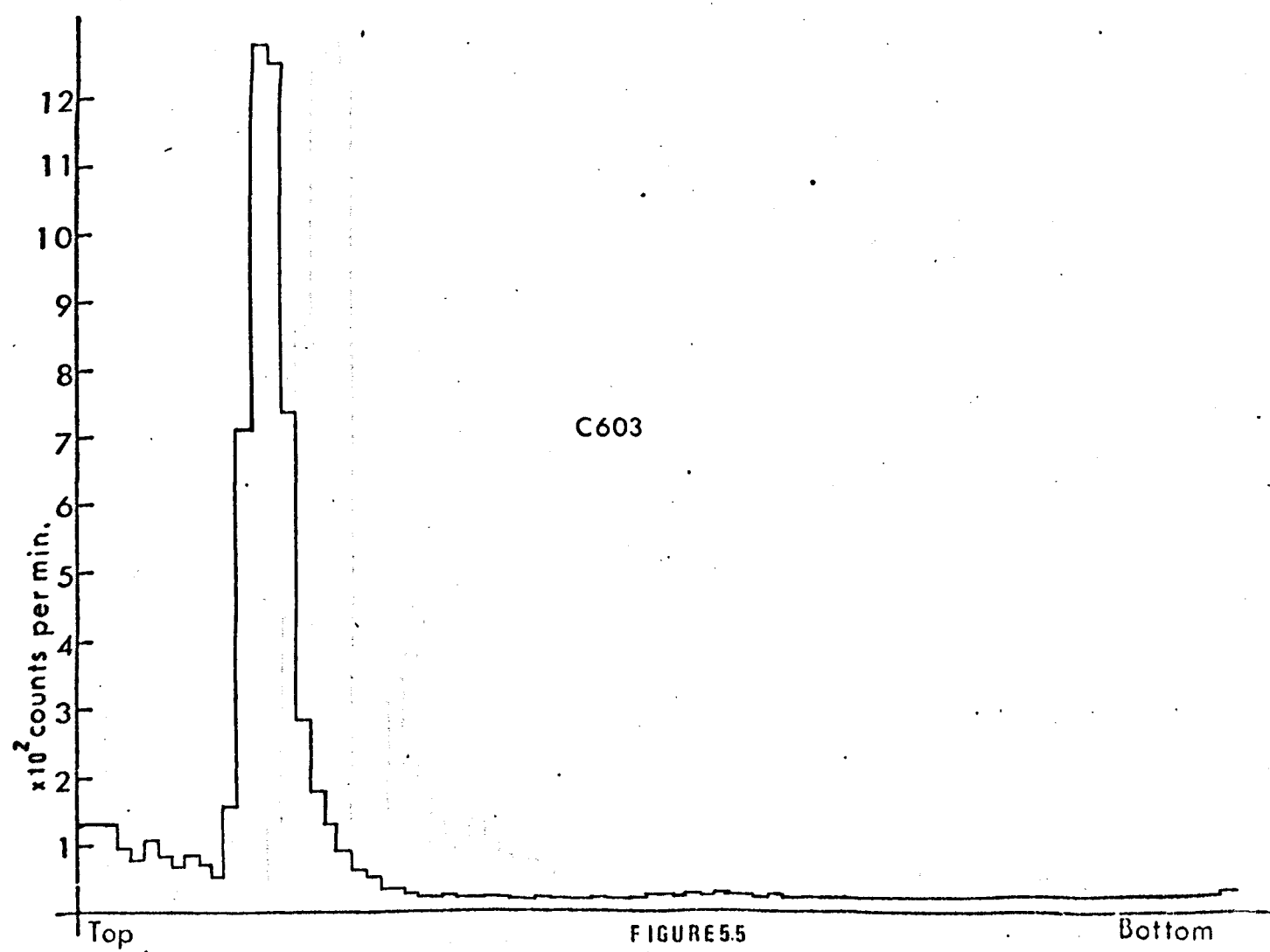
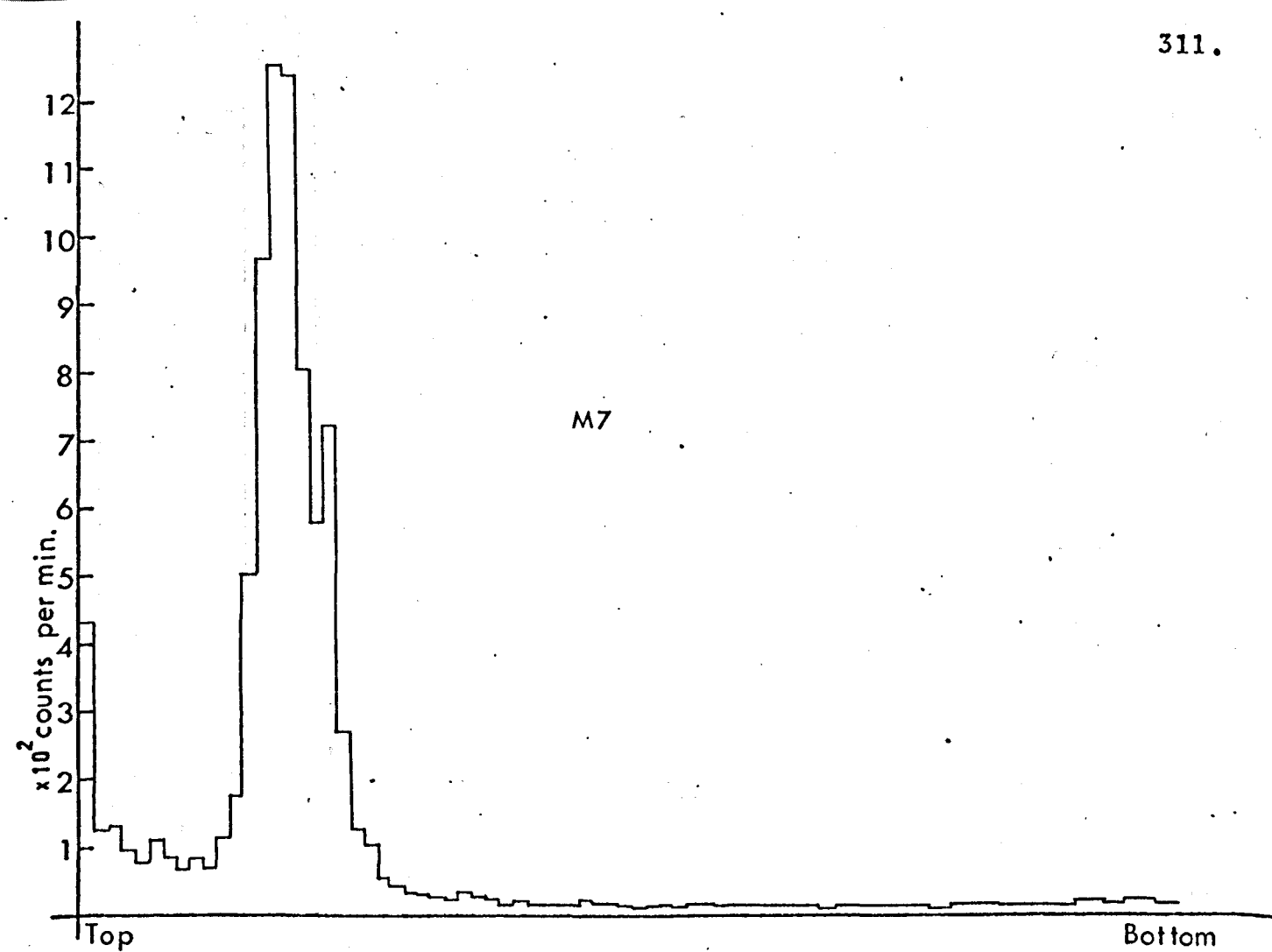


FIGURE 5.5

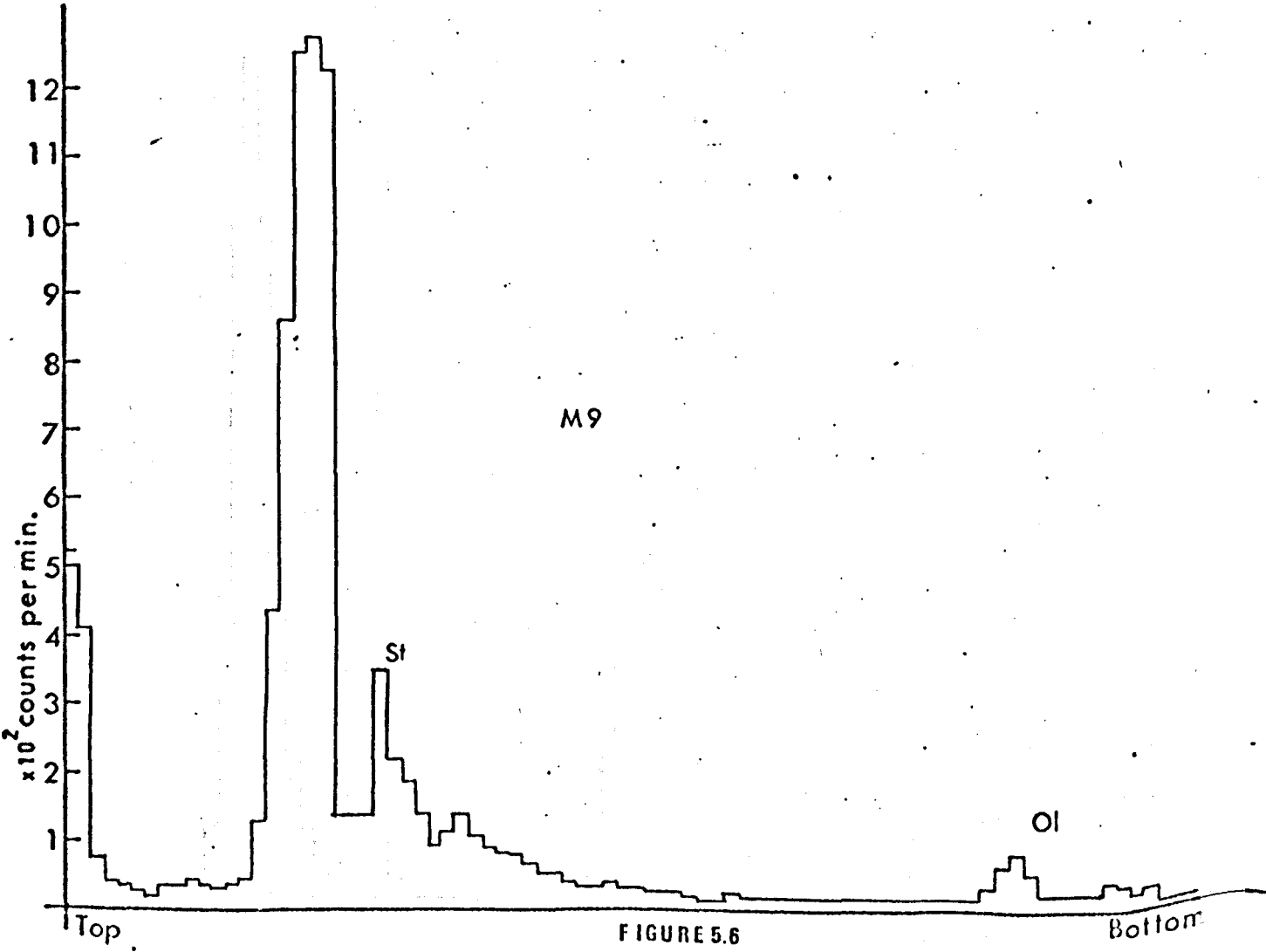
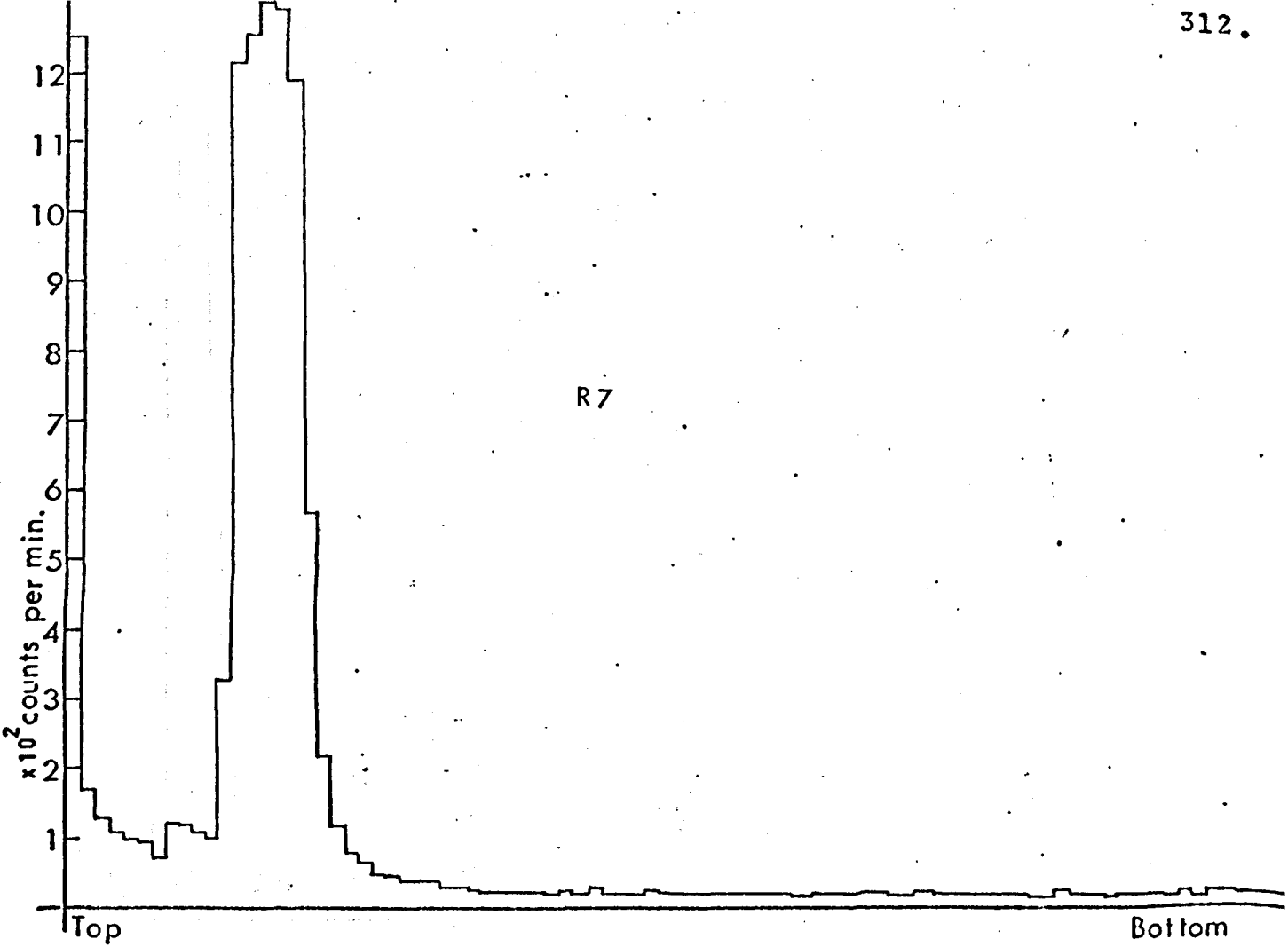


FIGURE 5.6

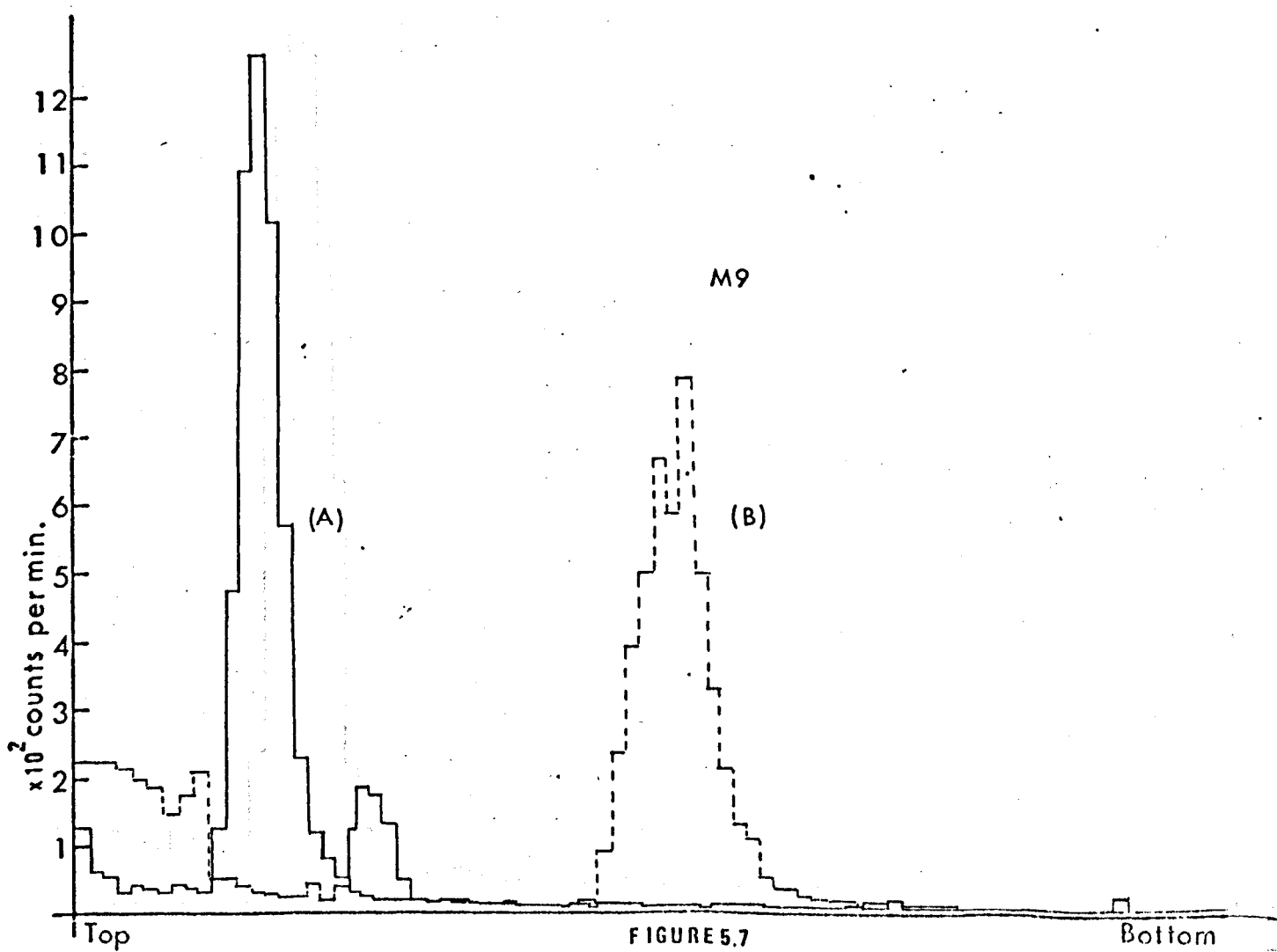
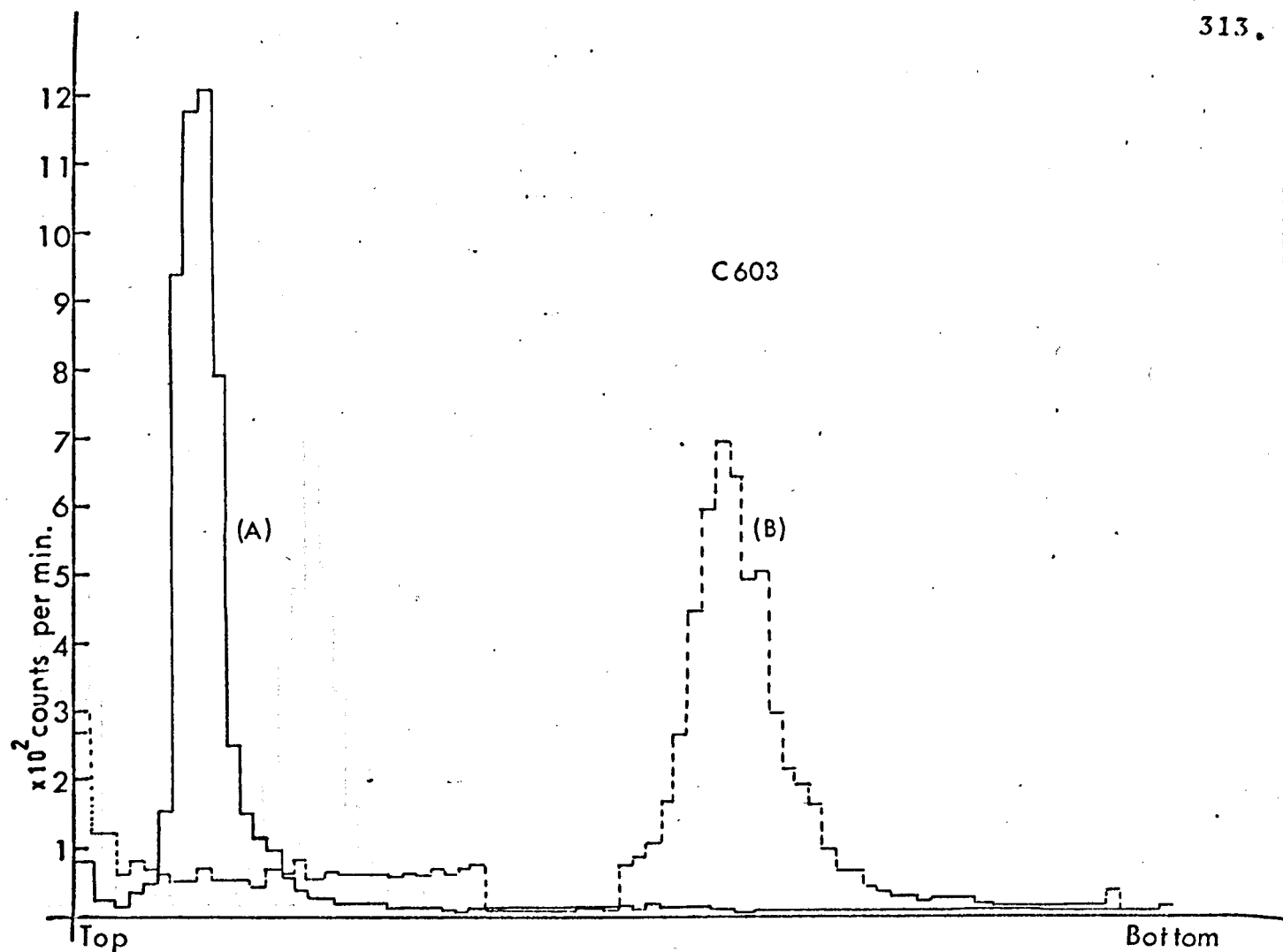


FIGURE 5.7

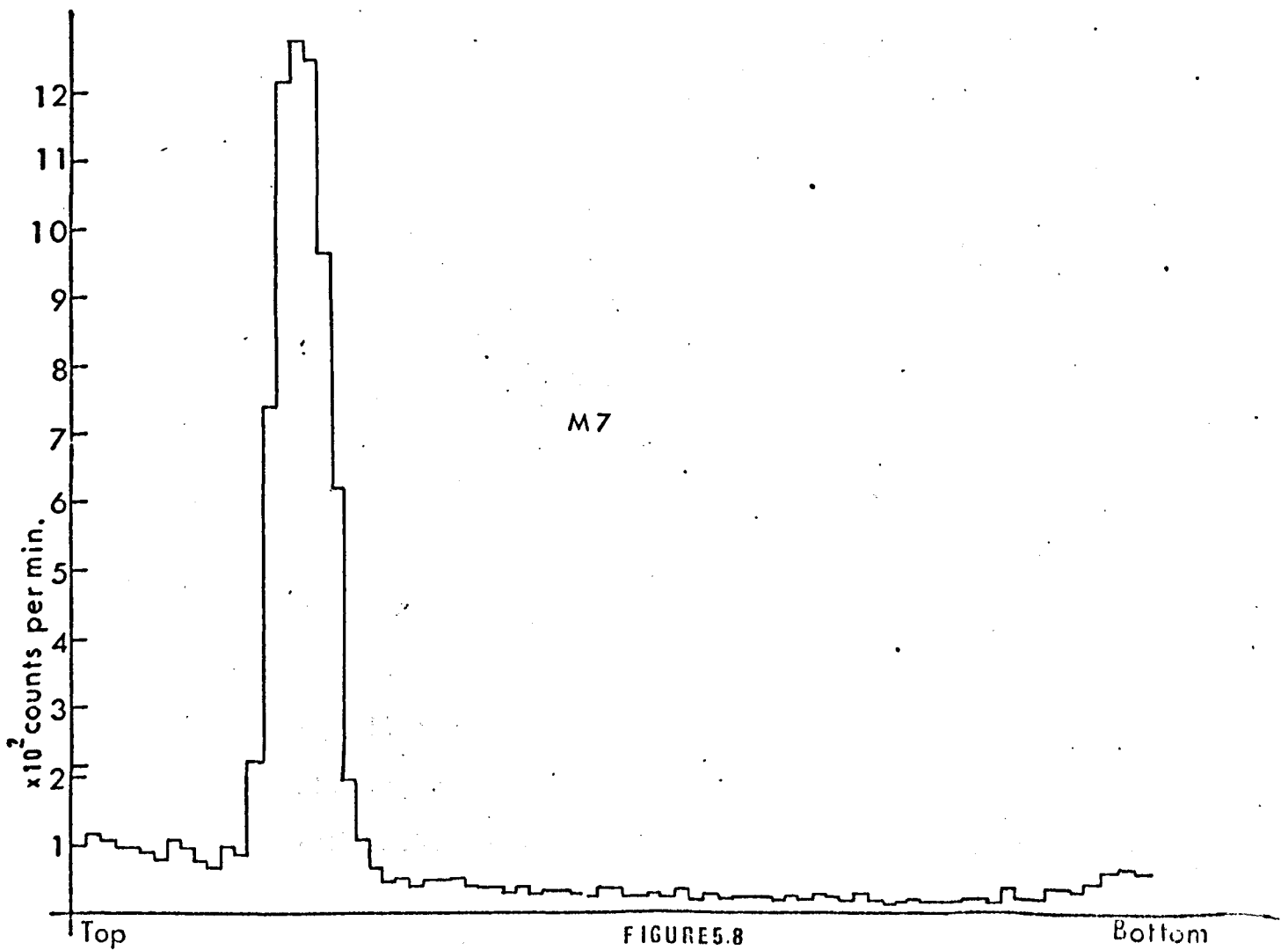
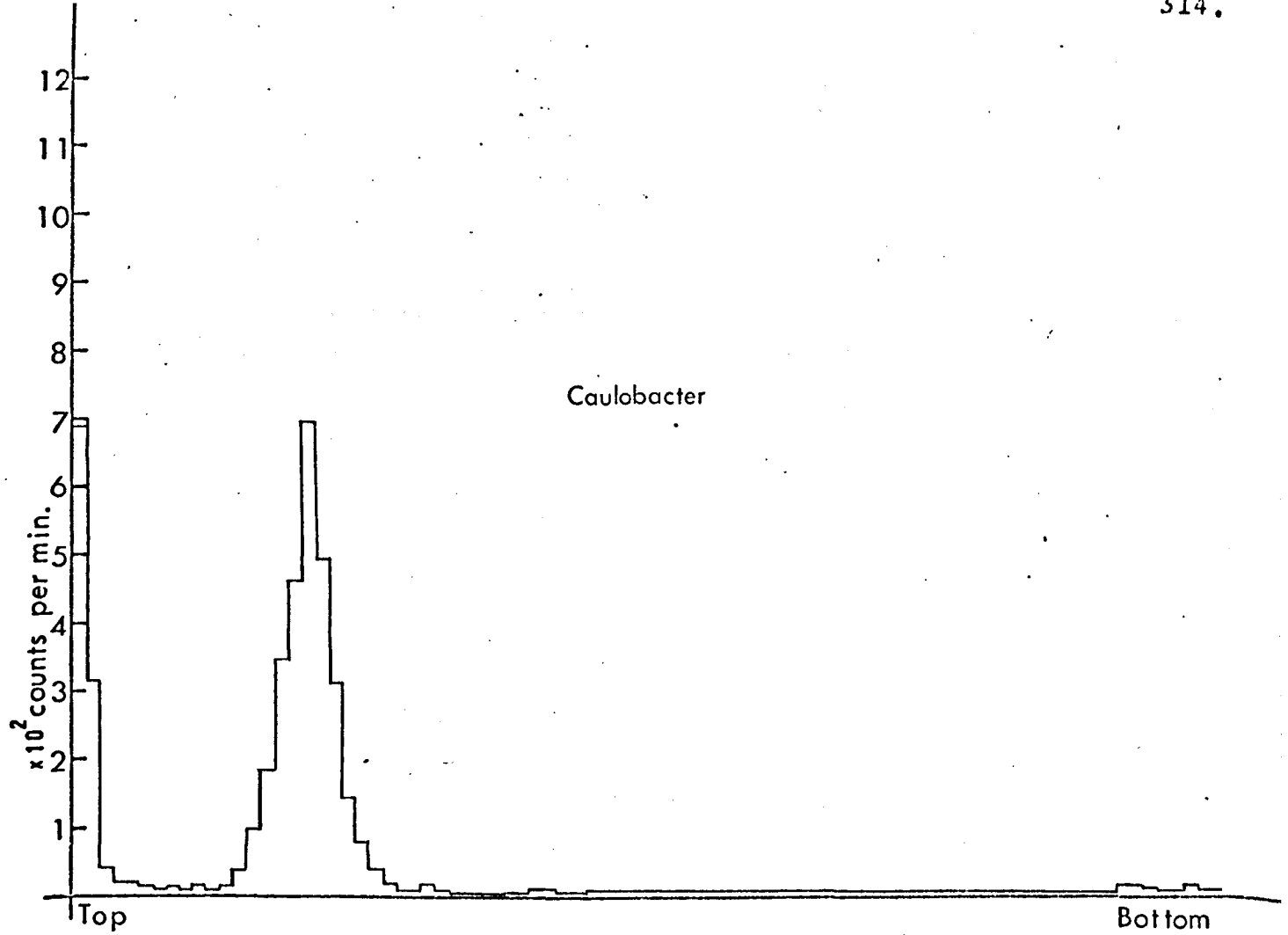
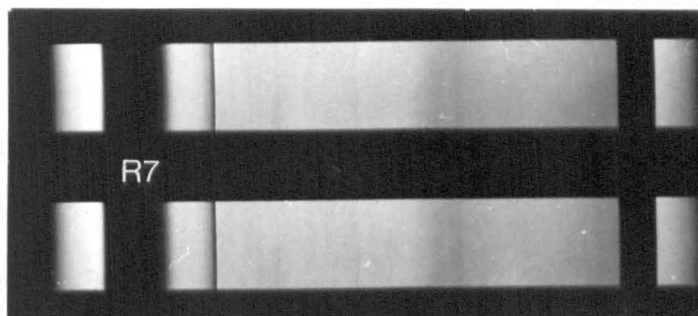
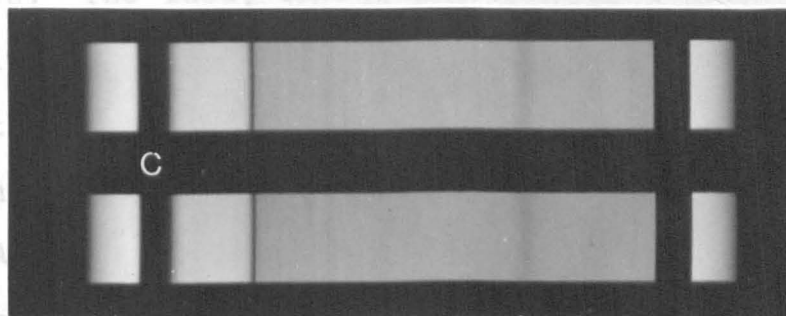


FIGURE 5.8



A



B

Plate 5.4

Equilibrium sedimentation pattern of E. coli strain R7 (A) and Caulobacter DNA (B) in alkaline (pH 12.5) CsCl density gradients.

thymine content. Alternatively, the discrete bands may be double stranded circular plasmids initially masked in neutral CsCl density gradients by linear double stranded DNA. Since strand separation was not successful with E. coli strain C603, which contains no free plasmids, the latter is favoured.

The Caulobacter DNA yielding only a single species under alkaline conditions is correspondingly explained in two ways. Either both linear DNA strands have the same guanine and/or thymine content or there are no plasmids to be revealed.

The equilibrium sedimentation experiments taken in conjunction with the agarose:ethidium bromide and agarose:tritium electrophoretic systems, all failed to show the presence of satellite DNA in the Caulobacter isolates examined. The idea, therefore, that the reported Caulobacter satellite DNA (Shapiro et al, 1970) is functional in the obligatory differentiation process of this organism seems very unlikely.

A possible explanation of the identity of the satellite DNA reportedly only being present in stalked Caulobacter cells comes from studies on Caulobacter DNA replication and isolated Caulobacter DNA bacteriophages.

Degnin and Newton (1972) have shown that DNA synthesis is characteristic only of the stalked cells of Caulobacter crescentus i.e. in swarm cells the onset of chromosome replication is delayed until stalk synthesis has occurred.

DNA bacteriophages have been reported by a number of authors for C. crescentus (Poindexter et al, 1967; Agabian-Keshishian and Shapiro, 1970; Jollick and Gerencser, 1973) the majority of which infect one cell type specifically, most probably by selective attachment to unique cell wall receptor sites. Jollick and Gerencser (1973) have additionally shown abortive bacteriophage infection in Caulobacter.

Considering these observations in the 'satellite' context, it does not seem unreasonable to suppose that the satellite band encountered by Shapiro et al (1970) is a manifestation of 'phage plasmid replication occurring specifically in the stalked cell and not in the swarm cell; this would be in line with control of the chromosome replication system of Caulobacter. There are undoubtedly other explanations for the presence or absence of plasmid DNAs in bacterial systems but to account for the quantitative aspects and specificity of that in Caulobacter, the above explanation seems most likely, discounting the presence of DNA from a contaminant, not unknown as the source of 'satellite' species of DNA (Mandel et al, 1971).

Satellite (plasmid) DNA in other differentiating budding bacteria.

The obligate differentiating budding bacteria, Rhodomicrobium (Rm5), Rhodopseudomonas palustris, Rh. viridis, Rh. acidophila, Hyphomicrobium and Nitrobacter were screened by neutral CsCl density gradient sedimentation for the presence of satellite or plasmid DNAs.

DNA from each organism was assayed three times at varying sample concentrations (figure 5.9) so alleviating to some extent the problems of masking and dilution of the presumptive plasmids.

Figure 5.9

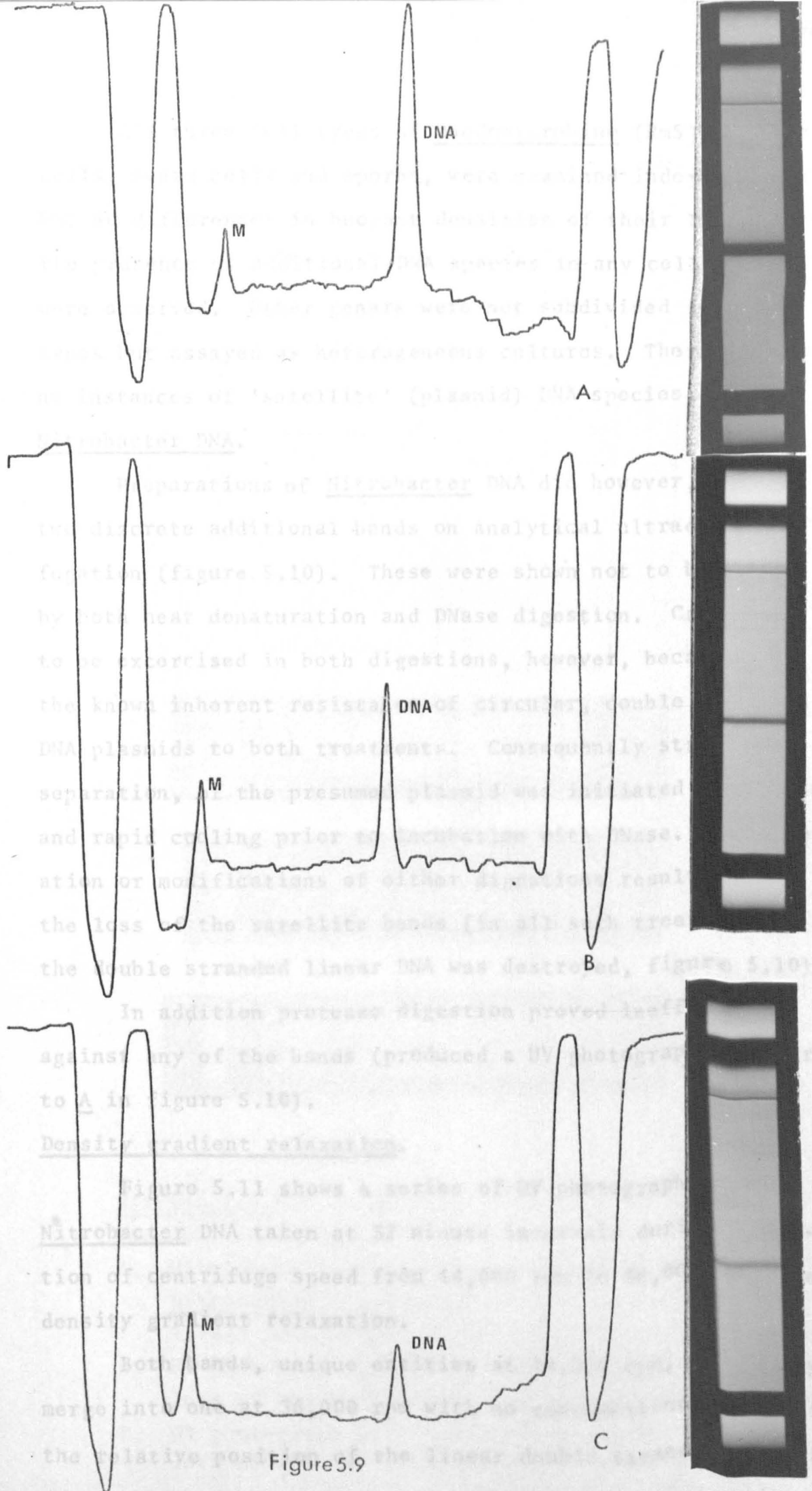
Microdensitometer tracings and UV absorption photographs of Rhodomicrobium (Rm5) DNA at three sample concentrations.

A. 2 μ g DNA.

B. 1 μ g DNA.

C. 0.5 μ g DNA.

(M meniscus)



All three cell types of Rhodomicrobium (Rm5), mother cells, swarm cells and spores, were examined independently but no differences in buoyant densities of their DNA or the presence of additional DNA species in any cell type were observed. Other genera were not subdivided into cell types but assayed as heterogeneous cultures. There were no instances of 'satellite' (plasmid) DNA species.

Nitrobacter DNA.

Preparations of Nitrobacter DNA did however, give two discrete additional bands on analytical ultracentrifugation (figure 5.10). These were shown not to be DNA by both heat denaturation and DNase digestion. Care had to be exercised in both digestions, however, because of the known inherent resistance of circular, double stranded DNA plasmids to both treatments. Consequently strand separation, of the presumed plasmid was initiated by heating and rapid cooling prior to incubation with DNase. No alteration or modifications of either digestions resulted in the loss of the satellite bands (in all such treatments the double stranded linear DNA was destroyed, figure 5.10).

In addition protease digestion proved ineffective against any of the bands (produced a UV photograph similar to A in figure 5.10).

Density gradient relaxation.

Figure 5.11 shows a series of UV photographs of Nitrobacter DNA taken at 32 minute intervals during reduction of centrifuge speed from 44,000 rpm to 36,000 rpm i.e. density gradient relaxation.

Both bands, unique entities at 44,000 rpm, gradually merge into one at 36,000 rpm with no concomitant change in the relative position of the linear double stranded DNA band.

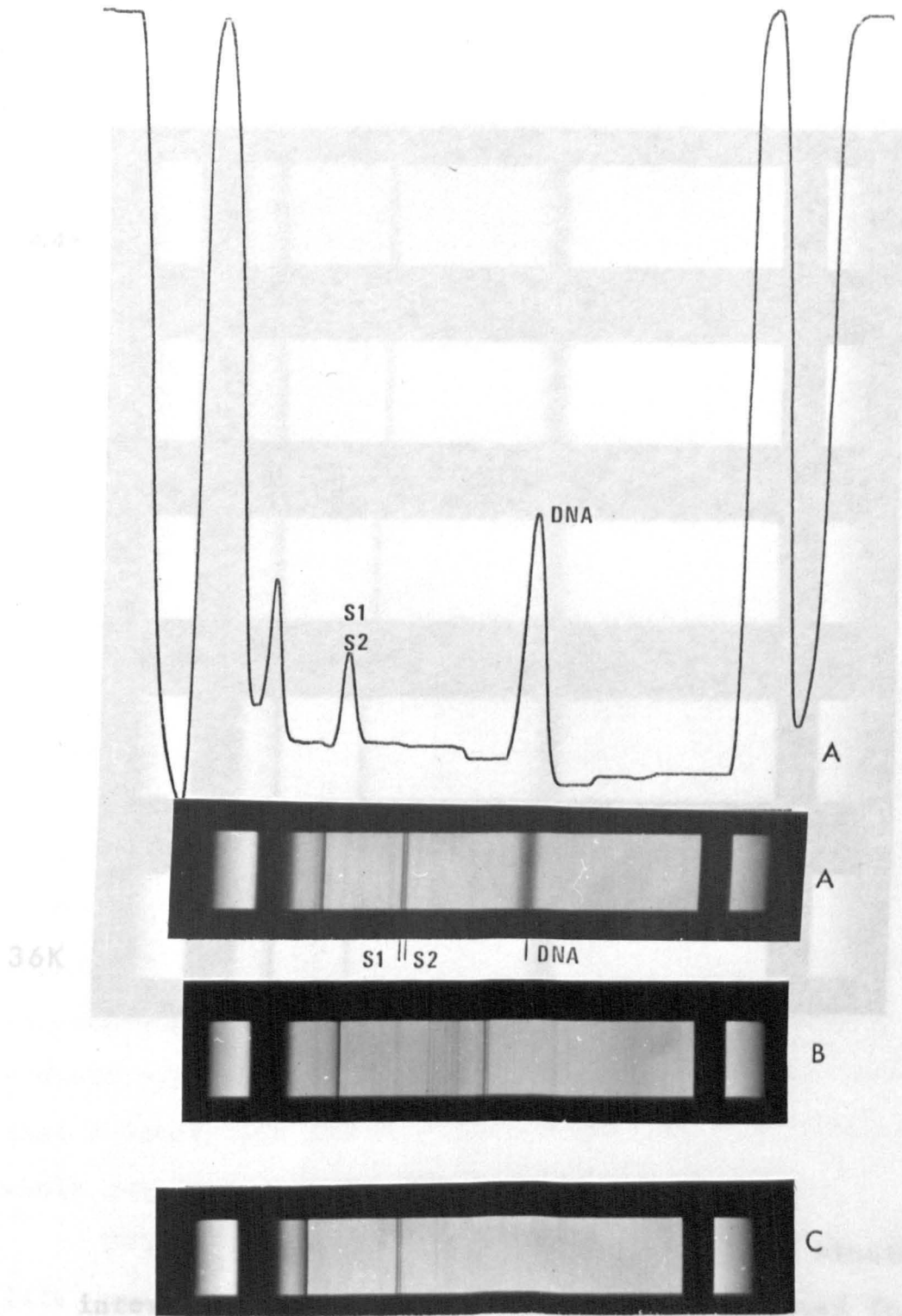


Figure 5.10

Nitrobacter DNA as analysed by neutral CsCl density gradient sedimentation.

- A. Microdensitometer trace and UV photograph of Nitrobacter DNA in neutral CsCl of mean density 1.7203 g/cm^3 . DNA- 1.7234 S_1 - 1.7149 S_2 - 1.7150
- B. UV photograph of heat denatured Nitrobacter DNA; loss of DNA band with retention of both satellite
- C. UV photograph of DNase digested Nitrobacter DNA, ^{species} loss of DNA band with retention of both satellite band

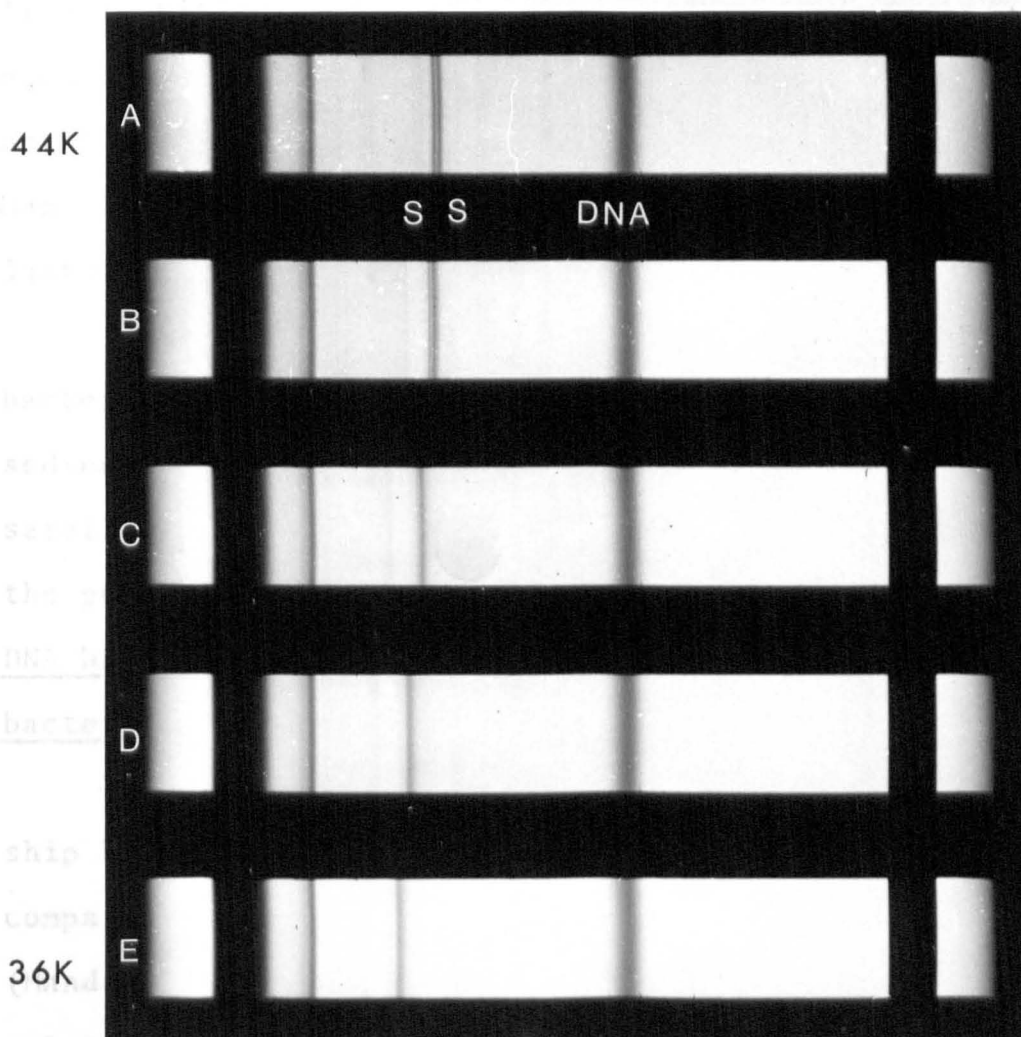


Figure 5.11

Sequence of UV photographs taken at 32 minute intervals during reduction in centrifuge speed from 44,000 rpm to 36,000 rpm. i.e. density gradient relaxation of Nitrobacter DNA and associated satellites in neutral CsCl of mean density 1.7203 g/cm^3 .

These experiments show the two satellite bands to be 'real' entities, not artifacts of cell or cell assembly each with uniform repetitive molecular weights. They are not DNA or protein but are most probably polysaccharide. Mandel (1968) comes to a similar conclusion in related instances.

In summary, the DNAs of obligate life cycled budding bacteria were examined by neutral CsCl buoyant density sedimentation. The buoyant densities were calculated. No satellite (plasmid) DNA species were encountered in any of the genera studied.

DNA base compositions of the obligately life cycled budding bacteria.

It has become well established that the interrelationship between a group or groups of microorganisms can be compared by the overall base composition of their DNA content (Mandell, 1966) i.e. for bacterial species to be closely related it is necessary that their DNAs should have a similar overall base composition. This method has the advantage that a comparison can be made directly on the basis of the whole genome.

DNA was extracted from various species of obligate life cycled budding bacteria and the base composition of each examined (at least twice using different marker DNAs) (figure 5.12) by CsCl density gradient centrifugation.

The buoyant densities and the corresponding base compositions of the various DNA preparations are listed in table 5.3 in addition to those already reported in the literature (the prefixed genera are those reported in the literature). In general, the value of the buoyant density

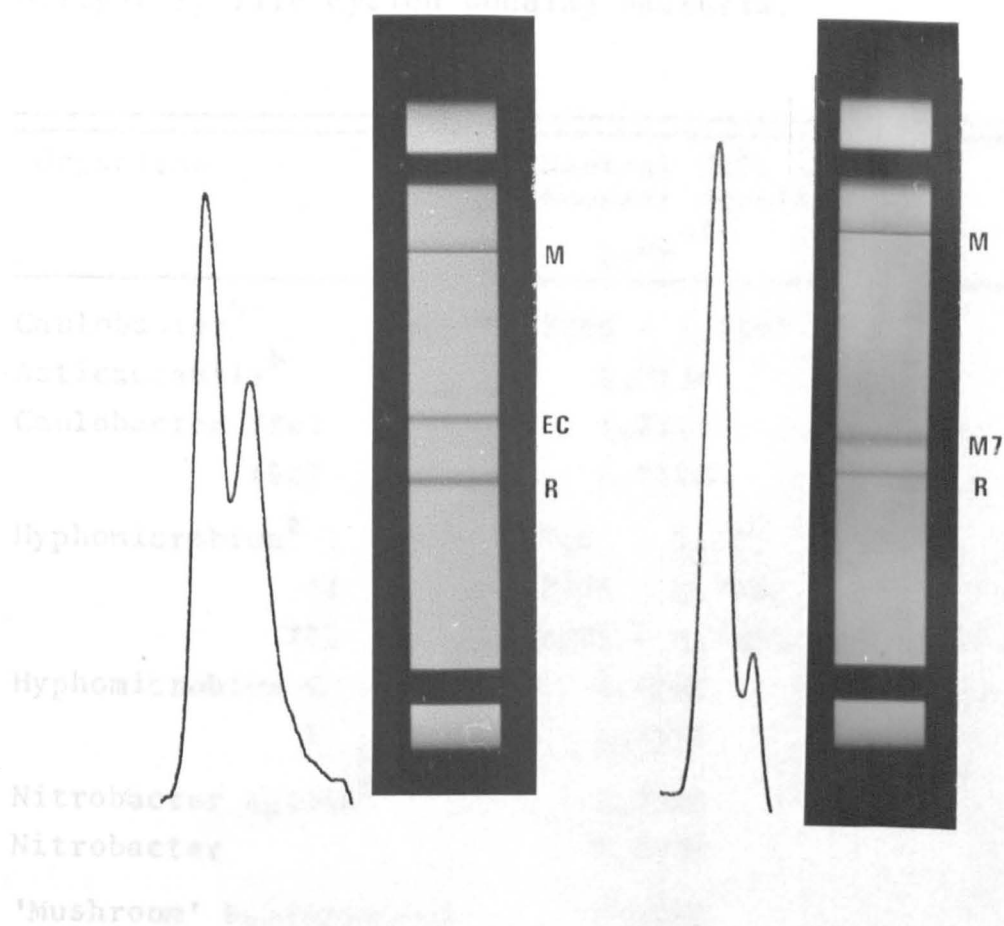


Figure 5.12

Rhodomicrobium (Rm5) DNA in relation to two known DNA markers, *E. coli* (M7) DNA and 'phage EC₁ DNA.

Rhodopseudomonas palustris

R. palustris (PA)

Rhodopseudomonas viridis

R. viridis (OG)

R. acidophila^o

R. acidophila (PA)

R. acidophila (A)

Table 5.3

DNA-CsCl buoyant densities and base compositions of the obligatory life cycled budding bacteria.

Organisms	Neutral CsCl buoyant density ^a g.cm ⁻³	G+C(mole%)
<i>Caulobacter</i> ^b	1.7208 - 1.7257	62-67
<i>Asticacaulis</i> ^b	1.7139	55
<i>Caulobacter</i> (8c)	1.7215	62.7
(5c)	1.7197	61
<i>Hyphomicrobium</i> ^c I	1.718 - 1.721	59.2-62.2
II	1.7225 - 1.7235	63.8-64.8
III	1.7245 - 1.7255	65.8-66.8
<i>Hyphomicrobium</i> C	1.7201	61.3
X	1.7213	62.6
<i>Nitrobacter agilis</i> ^d	1.7237	65
<i>Nitrobacter</i>	1.7234	64.7
'Mushroom' bacterium(w)	1.6973	38.1
(G)	1.6909	31.5
<i>Rhodomicrobium</i> ^e <i>vannielii</i>	1.7205 - 1.7225	61.8-63.8
Type strain (ATCC 17100) ^e	1.7215	62.8
<i>Rhodomicrobium</i> (Rm5)	1.7217	63
<i>Rhodopseudomonas</i> ^e <i>palustris</i>	1.7235 - 1.725	64.8-66.3
<i>R. palustris</i> (Rp1)	1.7237	65
(Rp2)	1.7244	65.7
<i>Rhodopseudomonas</i> ^e <i>viridis</i>	1.7250 - 1.7300	66.3-71.4
<i>R. viridis</i> (OG)	1.7297	71.0
<i>R. acidophila</i> ^e	1.721 - 1.7255	62.2-66.8
<i>R. acidophila</i> (PA)	1.7237	65
(A)	1.7227	64

is reliable to $\pm 0.001 \text{ g.cm}^{-3}$, the probable maximum error in base composition therefore being $\pm 1\%$ G+C (Schildkraut, Marmur and Doty, 1962).

The DNA mole % G+C content of the bacterial genera under study all fall into the range 59 to 71% G+C, with the exception of Asticacaulis, 55% (only one species reported in the literature) and the 'mushroom' bacteria, 31.5% to 38% G+C. Subdivision of individual genera into categories or groups is possible, as proposed by Mandel et al (1971) for Hyphomicrobium. However, such procedures are dubious since individual species from each genus can be selected in such a fashion to give G+C mole percentages which can be assigned to any group or subdivision.

The genera of most interest in this thesis, the obligatory life cycled phototrophs, morphologically and physiologically very closely related, may have their unique characteristics reflected in the similarity of their G+C mole percentages. However, there are numerous bacterial genera which bear no relationship to the above but which have very similar G+C mole percentages (Handbook of Biochemistry, 1970). The value of such comparisons is therefore questionable.

The low G+C% content of the 'mushroom' bacteria are well out with the data published to date for Gram -ve bacteria and only show a close relationship to the G+C mole percentages published for mycoplasmas. From the physiological and morphological parameters however, (Whittenbury and Nicol, 1971) it is unlikely that there is any affiliation between mycoplasmas and the 'mushroom' shaped bacteria.

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